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EFFECT OF CARCINOGENS ON SMALL FREE-LIVING ORGANISMS
I. EBERTHELLA TYPHI

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INTRODUCTION

Up to the present time investigators are generally agreed that no observations have been made which prove conclusively that there is any constant and specific differences in chemical composition, in enzyme content, or in metabolism between normal and cancer cells of the same tissues. It is believed that a systematic study of the action of carcinogenic agents upon free-living, single-cell, and small multicellular forms will ultimately throw much light upon the mode of action of these same agents upon mammalian tissues.

In the process of development of individual multicellular organisms, biologists know as yet very little about the conditions or laws that control the growth, organization, and differentiation of cells.

Assuming that the cancer process, as often stated, is an abnormal type of growth or a disorganization, or even a dedifferentiation, then the central problem in the causation of cancer is to learn, if possible, under what conditions a normal mammalian cell obeying these unknown laws is transformed into a malignant cell possessing the power of uncontrolled and in-coordinated growth.

The statement is also frequently made that a cancer cell is a normal cell that has become permanently altered—has become, after long irritation, further differentiated—a somatic mutation. Of course, this concept does not provide us with a working hypothesis any more satisfactory than the statement that cancer is an abnormal type of growth, because the underlying mechanism of mutation is equally as obscure as the mechanism of carcinogenesis.

Nevertheless, there are good grounds for believing that all of the following processes—(1) growth, (2) organization, (3) differentiation, (4) the occurrence of mutations, and (5) carcinogenesis—are not unrelated. In fact, cancer can now be induced at will in suitable laboratory animals by the same physical agents (X-ray, ultraviolet light, and gamma rays of radium) that are known to increase the occurrence of biological mutations. Furthermore, the cancer process, like that of differentiation and mutation, is a more or less universal cell potentiality and is irreversible. Each cancer that arises spontaneously or that is induced artificially is different from every other cancer. Mutations reveal the same high degree of specificity.

Regarding the structure of cancer cells as compared with normal cells, the only vis-

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ible changes noted by the cytologist are certain abnormalities found in the nucleus and nucleolus. Even these changes are not specific for cancer and may be seen occasionally in normal cells. However, all these observations suggest that in cancer the genetic constitution of the cell has been affected. Cancer, then, may be a disease of chromosomes, and the response of chromosomes (organs of heredity) to sublethal external stimuli may be much slower than that of cytoplasmic material.

It is conceivable that that part of the cell (either chromosomal or cytoplasmic) which transmits specific characters to the daughter cells may reveal no detectable response to a stimulus for a considerable number of generations. At least, the full effect of this response may not manifest itself in the first few generations. The slow response of tissues to carcinogenic agents suggests that a mechanism of this kind may be present. Moreover, Earle and Voegtlin (*1*, *2*) have shown that normal tissue cultures *in vitro* did not respond to methylcholanthrene with any structural changes until about the sixtieth to eightieth day of growth. There were some immediate toxic effects and a slowing up of the rate of cell division.

It is reasonable then to assume that permanent structural or functional changes in cells require a long-continued stimulus extending over many generations—a chronic irritation of the germinal protoplasm through numerous cell cycles.

Regardless of the correctness of such concepts, it was with these theoretical considerations in mind that we decided to study the effect of two of the most powerful carcinogenic agents, one chemical (methylcholanthrene) and the other physical (gamma rays of radium), on the following selected, small free-living forms:

(1) *Eberthella typhi* (*Bacillus typhosus*)

(2) *Paramecium multimicronucleatum*

(3) *Stenostoma tenuicaudum*

In this paper the results upon *Eberthella typhi* are presented. The results upon the other organisms will be reported later.

PREVIOUS INVESTIGATIONS

Goldstein (*3*), using *Escherichia communior* and a simple synthetic culture medium, found that certain carcinogenic hydrocarbons (1,2,5,6-dibenzanthracene[1] and methylcholanthrene) accelerate the rate of reproduction of the test organisms. There were approximately 50 percent more organisms in the eighth and ninth hour of growth than in control cultures. With phenanthrene, a non-carcinogenic hydrocarbon, repeated tests showed curves identical with the controls. The author suggests a short microbiological test for carcinogenic hydrocarbons which might replace the tedious method available at present.

Dodge and Dodge (*4*) found that the morphology and growth of yeast (*Saccharomyces ellipsoideus*) is profoundly affected by methylcholanthrene with the production of giant cells and increased differentiation of cells within the colony. Total dry weight and fermentation are increased approximately one-third in saturated methylcholanthrene peptone-glucose solution.

Cook, Hart, and Jolly (*5*) reported an increase in yeast proliferation of about 50 percent with dibenzanthracene (9×10^{-4} molar), while anthracene in the same concentration had no effect.

Hopper and Clapp (*6*) produced evidence suggesting that there is a high degree of correlation between carcinogenicity of compounds and the stimulation of bacterial reproduction. They stated that the mechanism by which the carcinogenic compounds accelerate the growth of the

[1] *Hereafter referred to as dibenzanthracene.*

test organisms is quite obscure at the present time.

Mottram and Doniach (*7*) described experiments in which various cyclic hydrocarbons were tested on Infusoria. With few exceptions they found a correlation between the photodynamic and the carcinogenic activities.

Reimann and Hammett (*8*), employing multicellular forms (*Obelia geniculata*), demonstrated that dibenzanthracene stimulates the formation of more new hydranths than in control tests. Proliferation was accelerated; differentiation and organization tended to be enhanced. The authors believe that carcinogenic agents stimulate these expressions of developmental growth.

Wright and Anderson (*9*) studied the effect of dibenzanthracene on a mold (*Fusarium lini*). The water-soluble products of the compound not only increased the weight of mycelium but also gave a more rapid utilization of the glucose in the medium.

Hollaender, Cole, and Brackett (*10*) state that methylcholanthrene in the dark may stimulate the growth of yeast and that in the presence of near-ultraviolet radiation it has a toxic effect. By this biological method dilutions of methylcholanthrene can be detected down to 10^{-9}, whereas the fluorescence method will detect it down to 10^{-7}, and the chemical method down to 10^{-6}.

Mottram (*11*) showed that the cultivation of *Paramecium* for a long time in media containing 3,4-benzpyrene (1 part in 500,000, and 1 part in 1,000,000) produced abnormal forms in 62 and 122 days, respectively. The experimental cultures never underwent conjugation. He found that normal forms are often the progeny of abnormal forms and quickly outgrow the latter. Some of the abnormal forms, however, breed true.

MATERIALS AND METHODS

Our studies were designed to observe not only the effect of the carcinogenic agents upon single organisms or single cultures but also the effect upon the species after a continuous exposure over a long period of time and over hundreds and even thousands of generations.

In order to test the effect of methylcholanthrene and radium emanations upon the organism, *Eberthella typhi*, it was of course necessary to exclude absolutely any possibility of contaminating organ-

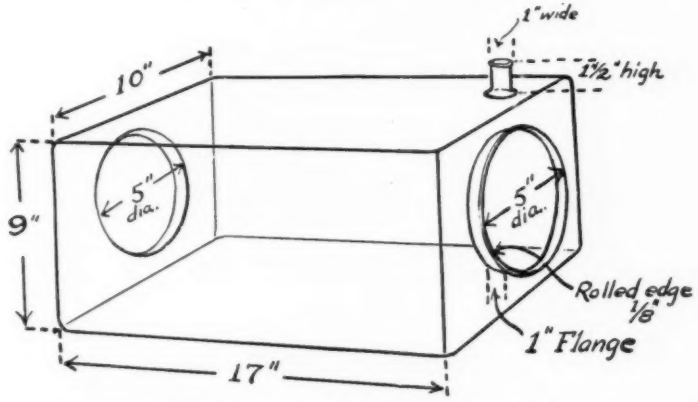

FIGURE 1.—Diagram showing dimensions of box.

isms. We have previously described (*12*) a pyrex glass box which was employed in this experiment (fig. 1).

Heat-resistant obstetrical gloves with long gauntlets are placed over the 5-inch flanges at each end after the necessary media, forceps, transfer needles, eye droppers, and rubber stoppers have been placed in the box. Experience has shown that it is necessary to place a small amount of cotton between the outer edge of the flanges and the rubber gloves, the gauntlet ends of which are stretched over the flanges and secured by elastic bands. The elasticity of the gloves alone is not sufficient to hold them in place when the hands are thrust in. The chimney at the top of the box is plugged with cotton, and the apparatus is ready for autoclaving at 15-pounds pressure for at least 1 hour on 3 successive days. Because of the entrapped air in the box, a long period of sterilization is apparently necessary even though a vacuum is first applied to the autoclave.

After the box has been sterilized and permitted to cool, one vial of the media is inoculated through the chimney. In doing this, care must be taken to bring the vial very close up to the chimney, and then the gloved hand which holds the vial to be inoculated must not be moved until after the cotton stopper of the chimney is again in place. Thus is prevented the suction of outside air into the box. After this first inoculation the box is never opened again until the last test vial has been inoculated.

In these tests we employed three metal racks holding 24 homeopathic vials each. Every vial contained 10 cc. of 1–5 diluted plain broth. The 24 vials of 1 rack were left unstoppered throughout the test as controls on the sterility of the air of the box. A transfer from the first inoculated vial was made to the second vial in 24 hours, and to a third vial in 48 hours. The fourth vial and all of the remaining vials of the 2 test racks contained 1 mg. each of commercial methylcholanthrene, purified by Dr. J. L. Hartwell of the National Cancer Institute staff.

In the test employing the emanations of radium, a 10-mg. platinum-iridium needle was transferred daily carrying over its own inoculum. The needle was 44.0 mm. long, 1.65 mm. in external diameter, and had a wall thickness of 0.5 mm. It has a gamma radiation equivalent to that from 10 mg. of radium element, according to the National Bureau of Standards certificate, when corrected to allow for the wall absorption. Platinum-iridium has a density of 21.5 and screens off approximately 99 percent of the primary beta radiation. After each tube had been inoculated in the case of the methylcholanthrene test, and after the radium needle had been transferred to the succeeding tube in the case of the radium experiment, the rubber stoppers were tightly fitted into the vials. All control vials, however, remained unstoppered until just before the box was dismantled.

RESULTS WITH METHYLCHOLANTHRENE

In this way *Eberthella typhi* was transferred in series through 240 vials each containing 1 mg. of methylcholanthrene. The test was started on March 15, 1939, and ended February 8, 1940, a period of about $10\frac{1}{2}$ months. Five boxes in series were used.

After dismantling the boxes, transfers were made by means of sterile tuberculin syringes, from every one of the 240 tightly stoppered vials to plain broth tubes, care being taken to sterilize the rubber stoppers with tincture of iodine before penetrating them with the sterile needle of the syringe; and from the broth tubes transfers were

made immediately by wire loop to a series of agar slants in order to obtain single colonies.

In no instance were any colonies observed that varied from the normal *Eberthella typhi*. Careful studies were made also on the morphology, staining, characteristics, motility, and the power to agglutinate antityphoid serum on organisms from various selected vials in the series. In addition, the organisms from the last vial of the methylcholanthrene-treated series (No. 240) were placed on 24 different sugars, and on litmus milk, gelatin, nitrate, and peptone broth. The production of acid and the fermentation reactions did not vary from those of the untreated *E. typhi*.

The only definite change noted was that the amount of visible growth in the vials of the latter end of the methylcholanthrene series (two hundredth to the two hundred and fortieth) was considerably less than in the vials of the first part of the series as determined by the degree of turbidity. In further support of this, it was also observed that a larger amount of material was needed in the inoculum from these latter tubes to obtain a good growth and distribution of colonies on agar slants.

RESULTS WITH RADIUM

Practically identical technique was followed in studying the effect of radium upon *Eberthella typhi*. The test was begun March 15, 1939, and ended January 16, 1940. Four boxes in series were used, also a 10-mg. radium needle, as previously described. The tests were carried through 192 successive transfers. Again the results were entirely negative. No reduction in the amount of growth was observed here as was noted in the methylcholanthrene test.

DISCUSSION

These tests suggest that *Eberthella typhi* displays no morphological, tinctorial, serological, or biochemical changes after being continuously exposed to methylcholanthrene and to the gamma rays of radium for 240 and 192 transfers, respectively. The strain (Rawlings) of typhoid organisms used has been a stock culture in the National Institute of Health for more than 30 years, and perhaps its stability may be owing to this long residence on practically the same laboratory medium.

It is conceivable that by using different amounts of the carcinogenic agents more definite results might have been obtained.

In this connection, it should be stated that we studied also the immediate effect of methylcholanthrene (1 to 1,000,000) on the rate of cell division of *Eberthella typhi* and obtained a definite though slight increase after 18 and 26 hours, as compared with control cultures and with cultures treated with phenanthrene. These tests are not recorded here in detail since they are merely confirmations of previous work by Goldstein on *Escherichia communior*.

In view of subsequent studies by us on the effect of these same carcinogenic agents on *Paramecium*, the negative results obtained above on a species of bacteria are extremely interesting. Methylcholanthrene has not only brought about an immediate stimulating effect upon the rate of cell division of the paramecia, but these organisms have displayed in addition a definite increase in survival period under conditions of partial starvation.

Radium-adapted paramecia have shown a striking and readily visible increased motility as well as a greater degree of light refraction. Such results were not detected in the first few transfers but were manifested very gradually. A detailed report of this study will be made later.

SUMMARY

After continuous exposure of *Eberthella typhi* to methylcholanthrene and to the gamma rays of radium for 240 and 192 consecutive transfers, respectively, no structural, functional, or immunological changes were observed other than an immediate stimulation of the rate of cell division which had been previously reported by other investigators. These essentially negative results, however, contrast strongly with the effect of the same agents upon a strain of *Paramecium*.

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FURTHER STUDIES ON THE SUSCEPTIBILITY OF HYBRID MICE TO INDUCED AND SPONTANEOUS TUMORS

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In a previous paper (*1*) it was recorded that reciprocal breeding between mice of strains C_3H and I and those of strains C_3H and Y produced hybrids which were of intermediate susceptibility to subcutaneously induced tumors when compared with the susceptibility of the parental strains. It was also noted that the hybrids derived by mating strains C_3H and I were more susceptible to both induced pulmonary tumors and spontaneous mammary cancer than were those derived by mating strains C_3H and Y. Such findings were of sufficient interest to encourage further investigation.

The purpose of this paper is to report additional observations on the susceptibility of hybrid mice to induced subcutaneous and lung tumors as well as to spontaneous mammary cancer. Other experiments along the same lines are in progress and will be reported in future papers.

DERIVATION OF HYBRID MICE

Eighteen young adult female mice of strain C_3H were mated to their brothers during March 1938. After weaning their first litter the C_3H females were mated to males of strain I or Y, and after weaning their second litter they were again mated to males of strain I or Y. The matings were arranged so that each C_3H female raised three litters, one by its brother, one by a strain I male, and one by a strain Y male. All hybrid mice thus obtained are designated as F_1 mice, and it should be noted that reciprocal matings were not made to procure the F_1 animals.

Mice of the appropriate backcross generations were procured by reciprocal breeding between the F_1 animals and mice of strains I, Y, or C_3H. Animals so obtained are designated as backcross mice.

The experimental animals lived under identical conditions. All received the same diet (Purina dog chow) and an unlimited supply of drinking water.

THE OCCURRENCE OF INDUCED SUBCUTANEOUS TUMORS

Only male hybrids were used to determine susceptibility to induced tumors. They were injected on October 11, 1938, when the F_1 mice were from 1 to 4, and the backcross mice were from 1 to 2 months of age. Each received a single subcutaneous injection in the right axilla of 0.8 mg. of commercial 1:2:5:6-dibenzanthracene [1] dissolved in 0.2 cc. of filtered lard.

The animals were examined at weekly intervals for the occurrence of tumors at the injection site, and as tumors appeared the mice were killed and their lungs examined carefully for the presence of macroscopic growths. This part of the experiment was terminated on September 18, 1939, or 48 weeks after injection, when all mice free from subcutaneous tumors were killed and autopsied. The time of appearance of subcutaneous tumors and the occurrence of induced pulmonary tumors are shown in table 1. The findings are presented as fractions in which the numerators denote the time of appear-

[1] *This sample was the same as that used in all previous experiments. Hereafter this compound will be referred to as dibenzanthracene.*

ance of subcutaneously induced tumors and the denominators the number of mice which had developed induced tumors of the lung.

TABLE 1.—*Time of observation of induced subcutaneous and pulmonary tumors in hybrid mice derived from strains C_3H, I, and Y*

[Numerator = number of mice developing subcutaneous tumors]
[Denominator = number of mice developing pulmonary tumors]

HYBRIDS FROM STRAINS C_3H AND I

Derivation of mice	Generation	Mice injected	Time in weeks											Mice killed and autopsied[1]	Total tumors
			18	21	24	27	30	33	36	39	42	45	48		
		Number												*Number*	*Number*
$C_3H \times I$	F_1	31	.....	1/1	2/1	4/4	7/7	4/4	1/1	1/1	2/2	.....	.....	9/9	22/30
$F_1 \times I$	I backeross	28	.....	2/0	.....	3/2	4/3	2/2	2/2	1/1	2/2	1/1	3/3	8/8	20/24
$F_1 \times C_3H$	C_3H backeross	24	1/0	2/1	4/4	1/1	3/3	2/1	5/4	2/2	1/1	1/1	.....	2/1	22/19
HYBRIDS FROM STRAINS C_3H AND Y															
$C_3H \times Y$	F_1	47	1/0	.....	8/0	4/0	7/1	6/1	4/1	3/2	1/0	4/4	1/1	8/5	39/15
$F_1 \times Y$	Y backeross	29	.....	1/0	1/0	5/4	3/1	3/2	2/1	2/2	1/1	2/1	.....	9/4	20/16
$F_1 \times C_3H$	C_3H backeross	64	.....	8/5	14/6	9/7	7/6	5/3	6/4	4/2	1/1	3/2	1/1	6/5	58/42

[1] All mice free from subcutaneous tumors were killed and autopsied Sept. 18, 1939.

It was shown (*2*) that C_3H mice when injected subcutaneously with 0.8 mg. of dibenzanthracene dissolved in lard develop induced tumors at the injection site in the average time of 18 weeks, while mice of strains I or Y are, on the average, at least twice as resistant to the same amount of the carcinogen. In table 2 in which the data of table 1 are summarized in a simplified form, it is seen that the F_1 hybrids were of intermediate susceptibility when compared with the parental strains since they were more resistant than the C_3H mice, and more susceptible than either I or Y animals.

TABLE 2.—*Occurrence of induced subcutaneous and pulmonary tumors following a subcutaneous injection of a lard solution of 0.8 mg. of dibenzanthracene into male hybrid mice derived from strains C_3H, I, and Y*

HYBRIDS FROM STRAINS C_3H AND I

Derivation of mice	Generation	Mice injected	Mice developing induced subcutaneous tumors	Incidence of subcutaneous tumors (A)	Average time of appearance (B)	Carcinogenic index $\left(\frac{A}{B} \times 10\right)$	Mice developing induced lung tumors	Incidence of lung tumors
		Number	*Number*	*Percent*	*Weeks*		*Number*	*Percent*
$C_3H \times I$	F_1	31	22	71	29.6	24	30	97
$F_1 \times I$	I backeross	28	20	71	33.2	21	24	86
$F_1 \times C_3H$	C_3H backeross	24	22	91	29.9	31	19	79
HYBRIDS FROM STRAINS C_3H AND Y								
$C_3H \times Y$	F_1	47	39	83	31.5	26	15	32
$F_1 \times Y$	Y backeross	29	20	69	31.5	22	16	55
$F_1 \times C_3H$	C_3H backeross	64	58	91	29.0	31	42	66

In the previous investigation (*1*) the F_1 hybrids were derived by reciprocal matings and consisted of both males and females. The experiment lasted for 38 weeks during which time 72 percent of the $C_3H \times I$ hybrids and 75 percent of the $C_3H \times Y$ hybrids developed subcutaneous tumors. In the present experiment, 64 percent of the $C_3H \times I$ hybrids and 70 percent of the $C_3H \times Y$ hybrids had developed tumors within 38 weeks after injection. Thus, the present experiment confirms in this respect the findings of the earlier work.

The occurrence of induced subcutaneous tumors in the backcross mice, as summarized in table 2, indicates that reciprocal breeding between the F_1 hybrids and C_3H parental strain produced mice which were more susceptible than those procured by reciprocal crossing of the F_1 hybrids to either strain I or Y. There was no striking difference between the average time of appearance of tumors in the four backcross groups, but 91 percent of the $F_1 \times C_3H$ animals developed tumors as compared with 71 percent of the $F_1 \times I$, and 91 percent of the $F_1 \times C_3H$ as compared with 69 percent of the $F_1 \times Y$. Furthermore, the $F_1 \times C_3H$ mice were more susceptible than the F_1 hybrids. Such results in the hybrid generations suggest that genetic factors played some role in determining the degree of susceptibility of the mice to the action of the carcinogen and that these factors were probably of a dominant character. The difference in degree of susceptibility is best shown in the seventh column of the table where a modification of Iball's (*3*) carcinogenic index of the relative potency of carcinogenic compounds has been used to ascertain the relative susceptibility of the experimental animals. The index here is derived by dividing the tumor incidence in percent by the average latent period and multiplying by 10, and is useful for measuring the degree of susceptibility of mice of known ancestry to the action of a cancer-producing compound.

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THE OCCURRENCE OF INDUCED PULMONARY TUMORS

The incidence of induced lung tumors is also summarized in table 2. It is obvious that of the F_1 hybrids those derived from the $C_3H \times I$ cross were more susceptible than those obtained from the $C_3H \times Y$ cross. This result is also in harmony with the earlier findings (*1*) in which 88 percent of the $C_3H \times I$ mice and 13 percent of the $C_3H \times Y$ animals developed pulmonary tumors. Up to the thirty-eighth week of the present experiment, 95 percent of the $C_3H \times I$ and 15 percent of the $C_3H \times Y$ animals had developed lung tumors.

This pronounced difference in susceptibility of the F_1 hybrids cannot be explained by comparing the relative susceptibilities of the parental strains. In a previous investigation (*2*) 44 strain I mice were injected subcutaneously with 0.8 mg. of dibenzanthracene dissolved in lard; 48 weeks after injection 50 percent had developed lung tumors. This incidence is lower than the 97-percent incidence in the $C_3H \times I$ mice of the present experiment. There is little evidence concerning susceptibility of either strain C_3H or Y following subcutaneous injection of the hydrocarbon, for C_3H mice develop subcutaneous tumors before lung tumors appear and a relatively small number of Y mice have received a subcutaneous injection of the compound.

In order to check the results of subcutaneous injection, a similar investigation was performed in which the lungs of mice of strains I, Y, and C_3H, and their F_1 hybrids were tested for susceptibility to intravenously injected 20-methylcholanthrene.[2] The intravenous route of injection is more desirable than the sub-

[2] *Hereafter called methylcholanthrene.*

cutaneous route for precise determinations of lung-tumor susceptibility, since the amount of carcinogen entering the blood stream can be ascertained; the reaction is more marked than after subcutaneous administration; and tumors do not occur at the site of injection. Shimkin (*4*) has used this method for determining the susceptibility of various strains of mice by killing the animals at regular intervals after injection and counting the number of lung tumors in each positive pair of lungs. His findings as regards strains I, Y, and C_3H, together with the results obtained in F_1 hybrids are presented in table 3. All mice received a single intravenous injection of 0.5 mg. of methylcholanthrene dispersed in 0.5 cc. of horse serum saturated with cholesterol.

TABLE 3.—*Susceptibility of three strains of mice and their hybrids to a single intravenous injection of 0.5 mg. of methylcholanthrene*

Mice injected	Number	Tumor incidence 26 weeks after injection	Average number of tumors in positive lungs
		Percent	
I	[1] 16	75	[1] 3
Y	[1] 10	60	[1] 3
C_3H	[1] 13	45	[1] 2
$C_3H \times I$	11	100	5
$C_3H \times Y$	15	45	1.6

[1] Data from Shimkin (*4, p. 231*).

The results were similar to those following subcutaneous injection, for it was shown that the hybridization of C_3H and I mice produced offspring which were more susceptible to induced pulmonary tumors than either of the parental strains, while hybridization of C_3H and Y mice resulted in offspring which were at least as resistant as the more resistant of the parental strains.

The incidence of induced lung tumors in the four groups of backcross mice (table 2) indicates that the strain I animals were more susceptible to this type of tumor than those of strain Y, because those generations produced by mating I to C_3H were consistently more susceptible than those resulting from mating Y to C_3H. It is seen, however, that both $F_1 \times I$ and $F_1 \times C_3H$ were more resistant than the $C_3H \times I$ group and that both $F_1 \times Y$ and $F_1 \times C_3H$ were more susceptible than the $C_3H \times Y$ generation. These findings in the backcross generations tend to emphasize the striking differences in the F_1 generations discussed above. It is obvious that, from the genetic standpoint, modifying factors influenced markedly the degree of susceptibility of the F_1 hybrids to induced lung tumors. Previous investigations (*5*) in this laboratory indicate that all inbred strains of mice are susceptible to the induction of lung tumors and that susceptibility is a matter of degree only.

One factor controlling the degree of susceptibility is shown in the present study. By referring to the $C_3H \times Y$ mice (table 1), it is seen that 3, or 10 percent of the 30 mice which were necropsied during the first 36 weeks of the experiment, and 12, or 71 percent of the remaining 17 mice which were killed during the next 12 weeks, had developed lung tumors. This shows the importance of time in the induction of lung tumors in mice and suggests that certain genetic factors control the time of appearance of the tumors.

THE OCCURRENCE OF SPONTANEOUS MAMMARY TUMORS

Of the 18 female strain C_3H mice used as parents for the F_1 hybrids, 17, or 94 percent, developed spontaneous breast tumors at an average age of 8.2 months. One died free from tumor when 9.5 months old. Of 12 other C_3H females used to obtain backcross mice, all developed spontaneous breast tumors at an average age of 7.9 months. Five strain I females and 9 strain Y females used to procure backcross

mice died at the average age of 14.1 and 12.5 months, respectively, and none developed breast tumor. This is in accordance with previous tabulations (*2, 6, 7*) of the occurrence of spontaneous mammary cancer in these three strains.

In the earlier experiment (*1*) it was recorded that of 12 virgin females derived from mating C_3H females to I males, all developed spontaneous mammary tumors at an average age of 8 months, while of 8 virgin females obtained by mating C_3H females to Y males none developed similar tumors before the age of 13 months. Subsequent histories of these mice show that 7 developed spontaneous breast tumors at an average age of 16.3 months and 1 died free from tumor when 14.5 months old.

In the present experiment the C_3H females bore litters to their brothers, to an I male, and to a Y male. The $C_3H \times C_3H$ offspring were all mated because they were members of the colony of this strain propagated in this laboratory The offspring of the $C_3H \times I$ and $C_3H \times Y$ matings were separated into two groups—one of virgins, and one of breeders each of which raised one litter.

The occurrence of spontaneous mammary tumors in the F_1 hybrids is shown in table 4. The difference in degree of susceptibility is shown in the seventh column of the table where Iball's method has again been employed. It is obvious that hybrids born to C_3H mothers and I fathers were more susceptible than those born to the same C_3H mothers and Y fathers.

TABLE 4.—*The occurrence of spontaneous mammary tumors in female C_3H mice and in female hybrids derived from strains C_3H, I, and Y*

Derivation of mice	Group	Number	Mice developing spontaneous mammary tumors	Incidence of spontaneous mammary tumors (A)	Average time of appearance (B)	Carcinogenic index $\left(\frac{A}{B} \times 10\right)$	Mice dying without tumor	Average age at death
			Number	*Percent*	*Months*		*Number*	*Months*
$C_3H \times C_3H$	Breeders	54	54	100	8.1	123	0	----
$C_3H \times I$	Breeders	25	24	96	8.5	113	1	17.5
	Virgins	20	18	90	11.4	79	2	17
$C_3H \times Y$	Breeders	31	29	94	10.7	88	2	21
	Virgins	24	19	79	12.7	62	5	19.9

It should be noted that in the former investigation (*1*), virgin females procured by breeding C_3H females and I males developed tumors at an average age of 8 months, while in this experiment the average age (11.4 months) at which tumors arose was approximately the same as in nonbreeding C_3H females (*7*). The reason for this difference is obscure. In the present study each C_3H female had nursed one litter of C_3H young before mating with an I or Y male. In the former investigation the C_3H females had not been bred before mating with I or Y males, and it was the offspring of their first mating which gave such striking differences in susceptibility. Such observations suggest that mice of the second or third litters born to C_3H females may not develop tumors as early as those of the first litter. This postulation is supported by the next experiment recorded in this paper in which the first litter virgin females born to C_3H females and I males developed tumors at the average age of 10.3 months. Perhaps the agent in mothers' milk, which is largely responsible for the occurrence of breast cancer in mice, diminishes in

amount with successive lactations or as the age of the mother increases. Experiments to test this possibility are now in progress.

Two other experiments are now in progress in which hybrid mice were obtained by mating strain C_3H females to males of other strains. The results of these experiments up to April 30, 1940, will be presented here since they present additional evidence of the influence of hybridization upon the occurrence of breast cancer in mice. In 1 investigation 12 female hybrids were obtained by mating C_3H females to strain I males, and 12 others by mating C_3H females to strain Y males. Both groups of 12 offspring were divided so that each contained 5 virgins and 7 breeders, each of which raised 1 litter. Of the 7 breeders obtained from C_3H mothers and I fathers, all developed breast tumors at an average age of 7.7 months; of 7 breeders from the $C_3H \times Y$ cross 4 developed breast tumors at an average age of 13.9 months, and 3 are living. Of the 5 virgins in each hybrid group, all of the $C_3H \times I$ developed tumors at an average age of 10.3 months and, of the $C_3H \times Y$, 2 developed tumors at an average age of 11.5 months; 1 that died when 11 months of age was free from tumor, and 2 are living. All living mice are 15 months old.

In the second experiment C_3H females were mated to males of strain I or C57 black. Each female hybrid was permitted to raise 1 litter. Of 26 hybrids obtained by mating C_3H females to I males, 22 developed breast tumors at an average age of 7.4 months, and the 4 survivors are 11 months old. Of 21 hybrids resulting from $C_3H \times$ C57 black cross, 12 developed tumors at an average age of 9.4 months, and the 9 survivors are 1 year old.

A summary of all four experiments in which female hybrids derived from mating C_3H females to males of strain I, Y, or C57 black is presented in table 5. In the colony of strain C_3H mice maintained in this laboratory the breeding females (*6*) (one litter only) and the nonbreeding females (*7*) develop breast tumors at an average age of 8.5 and 11.5 months, respectively. It is obvious, therefore, from the data shown in table 5 that the breeding of C_3H females to I males produces female offspring which are at least just as susceptible, and may be even more susceptible to spontaneous breast tumors than are strain C_3H females. It is also shown in table 5 that breeding C_3H females to Y or C57 black males results in female offspring which are considerably more resistant to breast tumors than are strain C_3H females.

TABLE 5.—*Occurrence of spontaneous mammary tumors in female hybrids derived by mating strain C_3H females to males of strains I, Y, or C57 black*

Derivation of mice	Group	Number	Mice developing spontaneous mammary tumors	Incidence of spontaneous mammary tumors (A)	Average time of appearance (B)	Carcinogenic index $\left(\frac{A}{B} \times 10\right)$	Mice dying without tumor	Average age at death	Mice living April 30,1940	Average age of living mice
			Number	*Percent*	*Months*		*Number*	*Months*	*Number*	*Months*
$C_3H \times I$	Breeders	58	53	91	7.9	115	1	17.5	4	11
	Virgins	37	35	95	10.1	94	2	17	.....	.....
$C_3H \times Y$	Breeders	38	33	87	11.1	78	2	21	3	15
	Virgins	37	28	76	13.5	56	7	17.9	2	15
$C_3H \times$ C57 black	Breeders	21	12	57	9.4	61	.....	.....	9	12

The outcome of the above-described investigations with F_1 generation mice suggests that genetic factors are involved in the occurrence of spontaneous mammary cancer in mice; for the male parent exerts some influence which determines in part, at least, the time of appearance of such tumors. Since tumors arose in the F_1 hybrids, it is also concluded that the susceptibility to this type of tumor in mice is inherited in a dominant manner but the number of genetic factors involved are unknown. The fact that a paternal influence is present suggests the activity of at least one modifying factor.

If the F_1 hybrids reveal the presence of genetic factors in the occurrence of spontaneous mammary cancer in mice, it might be expected that appropriate backcross animals would produce confirmatory evidence. Consequently, an experiment was designed so that backcross females could be used to determine (1) whether the paternal influence could be demonstrated in the backcross generations, and (2) whether the results obtained in the backcross generations would assist in the determination of the number of genetic factors involved in susceptibility to breast cancer. Bittner's work (*8*, *9*) reveals clearly the importance of mothers' milk in the occurrence of breast cancer in mice, and this factor must be taken into consideration when backcross animals are procured by reciprocal matings between F_1 hybrids and parental strains of high or low susceptibility. The C_3H strain of this experiment is highly susceptible to breast cancer, while the I and Y strains are much more resistant. Since all the F_1 hybrids were obtained by mating C_3H females to I or Y males, all were exposed equally to the milk influence. Furthermore, it may be assumed that all backcross mice derived from the F_1 females would also be exposed to the milk influence, for Bittner (*9*, *10*) has shown that hybrid mice transmit the milk influence to their offspring. Consequently, any variation in the degree of susceptibility of backcross mice obtained from either F_1 hybrid or C_3H females may be due to influences other than those found in their mother's milk.

The occurrence of spontaneous breast tumors in the backcross generations is shown in table 6 in which those derived from $C_3H \times I$ stocks are listed above and those derived from $C_3H \times Y$ stocks are listed below. In order to avoid the use of as many symbols as possible, the various backcross groups are designated as A, B, C, etc., in the table, and when possible will be referred to as such throughout the discussion. The backcross mice of each generation were divided into two groups: virgins and breeders. Each breeding mouse bore one litter which was killed within 24 hours after birth.

The first point of interest revealed in table 6 is the scarcity of tumors in backcross animals obtained by mating females of strains I or Y to F_1 hybrid males (B and F). It is also evident that backcross mice derived by breeding strain C_3H females to F_1 hybrid males (D and H) show a high incidence of breast tumors. This is further confirmation of the maternal (milk) influence in the development of breast tumors in mice.

It will also be noted that in every group of mice with the exception of backcrosses C and F, the breeding mice had a higher incidence of breast tumors than did the nonbreeding animals. This is shown clearly in the eighth column of the table where the carcinogenic index method of Iball has been applied.

The data in table 6 also reveal that mice procured by using strain I mice as a parental strain (backcrosses A, C, D) were consistently more susceptible to

TABLE 6.—*Occurrence of spontaneous mammary tumors in female backcross mice derived by mating F_1 hybrids to parent strains I, Y, and C_3H*

Derivation of mice	Backcross	Group	Mice used	Mice developing spontaneous mammary tumors	Incidence of spontaneous mammary tumors (A)	Average time of appearance (B)	Carcinogenic index $\left(\frac{A}{B}\times 10\right)$	Mice dying without tumor	Average age at death
			Number	*Number*	*Percent*	*Months*		*Number*	*Months*
BACKCROSS FROM $C_3H\times I$									
$F_1♀\times I♂$	A	Breeders	14	12	85.7	10.4	82	2	18.7
		Virgins	14	5	35.7	9.7	37	9	17.3
$I♀\times F_1♂$	B	Breeders	9	0	0	----------	0	9	17.7
		Virgins	8	0	0	----------	0	8	17.6
$F_1♀\times C_3H♂$	C	Breeders	6	5	83.3	10.0	83	1	19.5
		Virgins	5	5	100.0	9.7	103	0	---------
$C_3H♀\times F_1♂$	D	Breeders	6	6	100.0	7.2	139	0	---------
		Virgins	7	6	85.7	9.3	92	1	19.0
BACKCROSS FROM $C_3H\times Y$									
$F_1♀\times Y♂$	E	Breeders	21	2	9.5	13.5	7	19	17.2
		Virgins	18	1	5.6	8.0	7	17	16.3
$Y♀\times F_1♂$	F	Breeders	10	0	0	----------	0	10	15.6
		Virgins	11	2	18.2	10.5	17	9	16.0
$F_1♀\times C_3H♂$	G	Breeders	19	15	79.0	12.3	64	4	18.2
		Virgins	18	9	50	13.9	36	9	17.4
$C_3H♀\times F_1♂$	H	Breeders	16	15	93.8	11.6	81	1	19.0
		Virgins	17	7	41.2	12.4	33	10	17.7

mammary tumors than those derived from strain Y animals (backcrosses E, G, H). This difference was striking when F_1 hybrids were mated to males of the I (backcross A) or Y (backcross E) strains. The outcome in these groups may be regarded as confirmation of the results obtained in the F_1 hybrids, in that crossing strains C_3H and I produces hybrids which are more susceptible to breast tumor than those produced by crossing strains C_3H and Y.

Attention is directed to the higher degree of susceptibility in backcross mice derived by breeding F_1 females to C_3H males (backcross C) or C_3H females to F_1 males (backcross D) than in those obtained by mating F_1 females to strain I males (backcross A). Similar results attended the backcross animals obtained from strain Y as shown by the higher susceptibility of backcrosses G and H when compared with E. Obviously, backcross mice procured by crossing F_1 hybrids back to the C_3H strain were more susceptible than those resulting from crosses between the F_1 hybrids and I or Y strains. This may be regarded as further evidence that, if genetic factors were involved, they were inherited in a dominant manner since the C_3H strain is more susceptible to breast cancer than either the I or Y strains. The observation is in accord with the findings of Bittner (*9*) who used strains A and C57 black as test animals and concluded that susceptibility is inherited in a dominant manner.

The results in the various backcross generations fail to yield any information as to the number of genetic factors involved. The high incidence of 85.7 percent of tumors in the breeders of backcross A and the low incidence of 9.5 percent in comparable mice of backcross E (table 6) will illustrate the difficulty of attempting to apply Mendelian ratios to the results. The presence of modifying factors, as shown in this paper, would add to these difficulties. Indeed, the pronounced variation in susceptibility of

female mice, all of which were derived from the same strain C_3H females and therefore all exposed to the same milk influence, suggests that genetic factors exert their influence in the occurrence of spontaneous mammary cancer in mice by controlling the degree of susceptibility of the animals or the concentration of the extrachromosomal, or milk, influence.

DISCUSSION

During the past few years a series of investigations has been carried out with eight inbred strains of mice to determine the outstanding and comparative characteristics of the various strains relevant to tumor development, so that it can be known which strain supplies the most desirable test animal for any given phase of experimental cancer. It was shown (*2*) that the strains vary remarkably in their susceptibilities to spontaneous, induced, or transplantable tumors and that the investigation of the different types of tumors can be simplified by the use of an appropriate strain or strains.

In this study the hybridization of certain strains was carried out to ascertain whether susceptibility to three types of tumors was inherited according to genetic principles. Mice of strains C_3H, I, and Y were employed because they exhibit striking differences in their susceptibilities to induced subcutaneous tumors, spontaneous and induced pulmonary tumors, and spontaneous mammary tumors. Strain C_3H animals are highly susceptible to induced subcutaneous tumors and spontaneous mammary cancer but relatively resistant to pulmonary growths. Mice of strains I and Y are far more resistant to induced subcutaneous tumors and spontaneous breast tumors but are more susceptible to spontaneous or induced lung tumors than are strain C_3H mice.

Offspring derived from the three strains did not show any pronounced differences in susceptibility to subcutaneous sarcoma induced by dibenzanthracene. It is believed, however, that the findings justify the conclusion that susceptibility to this type of tumor was inherited in a dominant manner and that multiple genetic factors, or a mosaic of factors, were involved. This phase of the work dealt with a type of tumor which is of rare spontaneous occurrence in all three strains. Hence, it might be expected that any genetic factors involved would not consist of simple Mendelian ratios.

Since the relative susceptibilities of the three strains to induced lung tumors are known, the hybrids were better material for a study of inherited susceptibility to this type of tumor. Lynch (*11*) showed that susceptibility to induced lung growths is inherited in a dominant manner. Her observations were confirmed in this laboratory by using mice of strains A and C57 black, and in certain investigations evidence was adduced which suggested that a single dominant factor was responsible. In these particular experiments the mice received different amounts of dibenzanthracene subcutaneously (1.6 mg. in one experiment (*12*) and 1 mg. of the same carcinogen in another (*13*)) and were kept for 23 or 26 weeks after injection. It is now known (*5*) that all strains of mice are susceptible to the induction of pulmonary tumors and that the occurrence of such tumors depends upon the potency and amount of carcinogen injected, the route of injection, the period of time elapsing after injection, and other possible factors. Susceptibility to these tumors is a question of degree, and there is little doubt but that hybrids procured from strains A and C57 black would all prove susceptible under certain experimental conditions. The most important factor in the occurrence of induced lung tumors in

mice is the susceptibility of the test animal. The investigations of Lynch (*14*) and Bittner (*15*) demonstrate that the genetic constitution of the experimental animal is also of prime importance in the study of spontaneous pulmonary neoplasms in this species. Therefore, the induction of pulmonary tumors in the mouse is the result of the action of extrinsic influences upon the susceptibility of the animal and this intrinsic susceptibility is controlled by genetic factors.

In this investigation the genetic factors were controlled since the hybrids were derived from highly inbred strains. It was found, however, that F_1 hybrids obtained from mating strains C_3H and I were more susceptible to induced lung tumors than either parental strain, while those procured by mating strains C_3H and Y were no more susceptible than the more resistant parental strain. This shows that when inbred strains of mice of known susceptibility are crossed, it is not possible to predict the degree of susceptibility of the resulting hybrids. From the standpoint of genetics the findings suggest the presence of modifying or multiple factors or the summation of factors which lead to an accentuation or diminution in the degree of susceptibility.

The incidence of spontaneous mammary cancer in the hybrids confirms the observations of an earlier experiment (*1*). The hybrids derived by breeding C_3H females to I males were more susceptible than those obtained by breeding the same C_3H females to Y males. Indeed, the results suggest that the $C_3H \times I$ hybrids were more susceptible than the C_3H strain. On the basis of these observations of a paternal influence in the occurrence of mammary cancer in mice, it is suggested that any experiment in which hybrid mice are used for such studies demands adequate control animals.

Korteweg (*16*) noted this paternal influence in the occurrence of breast cancer in inbred mice. By mating females of the dilute brown strain to males of different genetic constitutions, he obtained hybrids which differed in susceptibility to breast cancer. He also drew attention to similar results in the published data of Murray and Little (*17*) and concluded that "the young ones undergo an influence from the paternal side" and that "this paternal influence we can scarcely imagine other than of chromosomal nature." Murray (*18*) has also demonstrated the influence of hybridization upon the occurrence of breast tumors in mice. The observations recorded here are in harmony with those of Korteweg and Murray and permit similar conclusions.

SUMMARY

Hybrids obtained from mice of strains C_3H, I, and Y were used to ascertain whether the tendency to develop induced subcutaneous and lung tumors as well as spontaneous mammary tumors have a genetic basis. The results permit the conclusion that susceptibility to all three types of tumor is inherited in a dominant manner and that multiple genetic factors, or modifying factors, are involved. It is also shown that when inbred strains of mice of known susceptibility are crossed, it is not possible to predict the degree of susceptibility of the resultant hybrids.

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THE INFLUENCE OF FOSTER NURSING UPON THE INCIDENCE OF SPONTANEOUS MAMMARY CANCER IN RESISTANT AND SUSCEPTIBLE MICE

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INTRODUCTION

It is generally accepted that in experimental animals the tendency to develop spontaneous tumors is inherited. The majority of earlier workers were inclined to agree that the mode of inheritance was in accordance with genetic principles but differed as to whether it was transmitted in a recessive or dominant manner. The reader is referred to Loeb's (*1*) review of this subject. There was, however, some difficulty in applying known genetic principles to the occurrence of all types of tumors, and Strong (*2*) in 1928 concluded that certain neoplasms in mice were probably due to nongenetic influences.

Data from various sources indicate that the tendency to develop mammary cancer in mice is due to nongenetic or extrachromosomal as well as genetic influences. Lathrop and Loeb (*3*), in 1918, while investigating the incidence of mammary tumors in mice stated (*3, p. 499*):

> However the fact that the mother strain prevailed in a much larger number of our cases than the father strain, and that several times (but not in all cases), in reciprocal crosses, the hybrids followed the tumor rate of the mother strain, suggests the possibility that as far as the hereditary transmission of mammary cancer in mice is concerned, the mother may be more potent than the father, and that perhaps under certain quantitatively varying conditions the mother strain may dominate over the father strain. This statement is merely a tentative conclusion at the present time and needs further investigation.

Definite information concerning the relative potencies of chromosomal and nonchromosomal factors in the development of tumors in mice was not available until the establishment of homozygous strains of animals capable of satisfying the requirements of carefully controlled genetic investigations. Such strains were developed largely through the efforts of Little and Strong. When reciprocal breeding was carried out between inbred strains exhibiting high and low incidences of breast cancer, it was found that offspring born to high-tumor-strain mothers developed breast tumors while those born to low-tumor-strain mothers remained relatively free from similar growths. Obviously, the maternal influence predominates in determining the development of breast tumors in mice. Murray and Little (*4, 5*) gave this problem extensive consideration and showed recently (*6*) that in first hybrid generations the extrachromosomal influence is 10 times as powerful as any chromosomal factors controlling the degree of susceptibility.

Bittner extended the problem by demonstrating that an extrachromosomal influence is present in the mother's milk. When newborn mice of a strain susceptible to breast cancer were suckled by foster mothers belonging to a resistant strain, the foster-nursed animals revealed a low incidence of breast cancers in spite of any inherited tendency toward their development. Bittner concluded that the absence of the milk influence resulted in a lowered incidence of breast tumors in susceptible mice but that its presence did not produce a significant increase of the incidence in resistant mice. As a resistant strain he

used mice of strain C57 black in which, according to Little, Murray, and Cloudman (*7*), only 0.5 percent of breeding females develop mammary cancer. In one paper (*8*) Bittner reported that 4 of 25, and in another (*9*) that 3 of 8 C57 black mice developed mammary tumors after nursing high-cancer females. Later (*10*) he recorded an incidence of 10.6 percent breast tumors in 104 C57 black mice fostered by high-cancer females and attributed the increase to sub-line variations within the C57 black strain. In a more recent publication (*11*) he reported an incidence of 11.3 percent of mammary tumors in 97 C57 blacks after foster nursing high-cancer females but concluded that "no significant increase in the breast tumor ratio was observed from fostering low-cancer stock young to high-cancer females."

It was found (*12*) in this laboratory that foster nursing of C57 black mice by high-tumor strain C_3H females raised the incidence of spontaneous breast tumors in the C57 blacks to 9 percent. In this experiment the fostered C57 blacks were permitted to raise but one litter. Bagg (*13*) also noted an increase in the number of breast tumors in C57 blacks after they had nursed upon strain C_3H, and Murray [1] obtained similar results by using females of the high-cancer dilute-brown strain as foster mothers. This similarity in the results of four investigators working in different laboratories is regarded as significant evidence that the milk of high-tumor-strain females contains an agent, or agents, contributory to the genesis of breast tumors in females of strain C57 black.

The object of this paper is to report two more experiments in which mice born to low-cancer-strain mothers were foster nursed by high-cancer-strain females and vice versa. It was shown (*12*) that the fostering of C_3H mice by C57 black females reduced the incidence of breast tumors in C_3H females, and, as recorded above, the ingestion of C_3H milk by C57 black young increased the incidence of breast tumors in the black strain. In view of these findings, it was considered advisable to ascertain (1) whether the ingestion of milk of other low-tumor-strain females would also lower the incidence of tumors in strain C_3H and (2) whether the ingestion of milk from strain C_3H females would produce breast tumors in females belonging to strains which were resistant to the spontaneous occurrence of this neoplasm.

EXPERIMENTAL PROCEDURE

Mice of strain C_3H, I, Y, C, and C57 black were used. All were raised in this laboratory and were descendants of litters originally procured from the Roscoe B. Jackson Laboratory. Strains C_3H, I, Y, C, and C57 black have undergone 25, 8, 9, 8, and 6 generations of inbreeding, respectively, since they were obtained from the Jackson Laboratory. Strain C_3H females are highly susceptible to the development of spontaneous mammary tumors (*14*) while those of strains C57 black, I, Y, and C are far more resistant (*15*). The animals were kept under similar conditions and on a diet of Purina dog-chow pellets, with an unlimited supply of water.

The first experiment was begun during October 1938, by brother-sister matings in all strains. Pregnant mice derived from these matings were examined at least twice daily to see whether they had given birth to litters. As the young were born, half of each C_3H litter was transferred to a foster mother of strain I, Y, or C; and as the I, Y, C, or C57 black mice were born, half of each litter was transferred to a C_3H foster mother. In this

[1] *Murray, W. S.: Personal communication.*

manner half of each litter was foster nursed, and half nursed by its own mother. This procedure gave litter-mate controls, and it is believed that the use of such controls is essential in this kind of investigations. After the mice were weaned, each female of strain I, Y, C, or C57 black was mated and permitted to bear three litters in rapid succession; that is, each litter was killed within 24 hours after birth. Bagg (*16*, *17*) demonstrated that rapid breeding and nonsuckling increase the incidence of breast tumors in mice and a modification of his method was employed here.[2] Each C_3H female bore one litter which was killed within 24 hours after birth. Experience has shown (*14*) that a single pregnancy is sufficient to produce breast tumors in practically all strain C_3H females at an average age of 8.5 months.

In the second experiment, begun during December 1938, C57 black females were mated to strain I males. When the young were born, half of each litter was foster nursed by a strain C_3H female and the remainder nursed by the mother. As in the first experiment, each female mouse was mated and bore three litters in rapid succession.

Previous work (*19*, *20*) suggested that the breeding of strain C_3H females to strain I males resulted in offspring which were more susceptible to spontaneous breast cancer than the parental C_3H mice. It was hoped that a comparison between the incidence of tumors obtained in the C57 black mice of the first experiment and the incidence in hybrids derived by mating C57 black females to strain I males would produce further evidence of the influence of hybridization upon susceptibility to spontaneous mammary tumors.

RESULTS

The results of the two experiments are presented in table 1. The time of appearances of tumors is listed in the table by periods of 2 months, but the figures in the column showing the average age at which the tumors occurred were computed from the number of tumors observed each week.

[2] *The results of Little and Pearson* (*18*) *do not exactly agree with those of Bagg.*

TABLE 1.—*Tumor incidence in high-breast-tumor-strain mice foster nursed by low-breast-tumor-strain mice and vice versa*

[Numerators=number of mice developing breast tumors]
[Denominators=number of mice dying free from tumor]

Experiment No.	Strain of mice	Nursed by—	Number	Number developing breast tumors or died free from tumor (in months)								Tumor incidence	Average tumor age	Average age at death without tumor	Mice living May 15, 1940	Average age of living mice
				5	7	9	11	13	15	17	Totals					
												Percent	*Months*	*Months*	*Number*	*Months*
1	C_3H	C_3H	15	....	4/0	6/0	5/0	....	....	....	15/0	100	8.4	....	....	....
	C_3H	C	12	....	....	1/0	1/1	3/0	....	1/0	6/1	50	11.8	9.5	5	15.8
	C_3H	Y	16	....	1/0	1/0	1/0	2/0	2/0	2/0	9/0	56	12.5	....	7	17.0
	C_3H	I	14	....	1/0	1/0	1/0	2/0	....	2/1	7/1	50	11.7	....	6	16.4
	I	I	7	....	0/2	0/1	0/4	....	....	....	0/7	....	....	10.6	....	....
	I	C_3H	6	....	....	0/1	0/1	1/3	....	....	1/5	17	11.5	11.1	....	....
	Y	Y	12	....	....	0/1	0/2	0/7	0/2	....	0/12	....	....	12.1	....	....
	Y	C_3H	14	0/1	0/1	1/0	1/1	0/4	0/5	....	2/12	14	9.0	12.0	....	....
	C57 black	C57 black	16	....	....	0/4	0/6	0/3	0/3	....	0/16	....	....	10.5	....	....
	C57 black	C_3H	14	....	2/1	0/4	0/5	0/2	....	....	2/12	14	5.7	9.7	....	....
	C	C	14	....	0/1	0/4	0/2	0/7	....	....	0/14	....	....	10.4	....	....
	C	C_3H	14	1/0	2/0	3/0	3/2	0/3	....	....	9/5	64	8.1	11.3	....	....
2	C57 black ♀ × I ♂	C57 black	21	0/1	....	0/2	....	0/5	0/2	....	0/10	....	....	11.0	11	13.0
	C57 black ♀ × I ♂	C_3H	17	1/0	6/0	3/0	1/0	1/2	....	....	12/2	71	7.8	11.7	3	13.0

Table 1 shows that foster nursing of C_3H young by females of strain C, Y, or I decreased the breast-tumor incidence in C_3H females from 100 to approximately 50 percent. Analysis of the results of this phase of the experiment confirmed the findings of previous work (*12*) in that the occurrence of breast tumors in fostered C_3H mice depends to a considerable extent upon the time the mice remain with their mother after birth. During the early part of the experiment the pregnant mice were examined at least twice each day to see whether their litters had been born. The last daily examination was made at 4 p. m. and the first on the following day at 9 a. m., except on Sundays or holidays when the period between examinations was 24 hours. Since the majority of litters were born during the night, this procedure limited the longest possible time most of the young were with their mothers to 17 hours. Tabulation of the results according to the longest possible time the newborn mice were with their mothers revealed that 42 C_3H young were fostered by females of strains I, Y, or C; 3 were with their mothers for 3 hours, and none developed a tumor; 32 were with their mothers for 17 hours, and 16, or 50 percent, developed tumors; and 7 were with their mothers for 24 hours, and 6, or 86 percent, developed tumors.

In the earlier experiment (*12*) tumors arose in 25 percent of C_3H young which were with their mothers for a possible 17-hour period before being fostered by C57 black females. This may be interpreted as suggesting that foster nursing of C_3H young by C57 black females was more effective in lowering the incidence of breast tumors in C_3H animals than fostering them upon females of strains C, I, or Y. Further analysis of the present experiment, however, showed that the 32 C_3H young which may have been with their mothers for 17 hours represented the mice from 17 litters; and of these, 11 mice from 8 litters all developed tumors, while of the remaining 21 mice from 9 litters only 5 developed tumors. This indicates that 8 litters of young were with their mothers for a longer period of time than the 9 in which few tumors developed. Hence, it is believed that a definite conclusion cannot be made concerning the relative efficacies of milk from females of the various strains in lowering the incidence of breast tumors in C_3H mice. Further investigations of this problem are in progress and will be reported later.

Table 1 also shows that breast tumors appeared in the fostered C_3H mice at a later average age than in nonfostered mice of the same litters. This observation is in harmony with previous findings (*12*) and indicates that the amount of milk obtained from C_3H females influences the time of appearance of breast tumors in fostered C_3H mice. There is a correlation between the amount of mothers' milk the C_3H young ingest during the early hours of life and the time of appearance of the tumors.

Table 1 also contains information concerning the occurrence of mammary tumors in resistant mice which were fostered by high-tumor-strain C_3H females. In experiment 1 a few breast tumors arose in mice of strains I, Y, and C57 black fostered by C_3H females and none in their litter-mate controls, but the results are considered inconclusive because the mice died at an early average age. The strain C mice of experiment 1 and the C57 black $\times$ I hybrids of experiment 2 gave significant results. It is seen that 64 percent of the fostered strain C mice and 71 percent of the fostered hybrids developed spontaneous mammary cancer while none of their litter-mate controls developed a similar tumor. Six litters of strain C mice were used in

experiment 1, and tumors arose in representatives of 4 of these, while of 10 litters used in the second experiment, 8 contained tumor-bearing mice. This demonstrates that sub-line differences within the strains propagated in this laboratory were not responsible for the results. Litter mates were used as controls in both experiments.

The early average age at which breast tumors occurred in the fostered strain C (8.1 months) and C57 black × I hybrids (7.8 months) was surprising. The mammary glands of these mice were apparently highly susceptible to the cancer-inciting influence in the milk of strain C_3H females.

Up to the present time the only strains of mice shown to possess the milk influence were those susceptible to the development of spontaneous mammary tumors. Bittner (*11*) claims that susceptibility of mammary glands to the milk influence has a genetic basis, and the experiments performed in this laboratory (*20*) support this conclusion. It is conceivable, however, that a strain of mice may possess the milk influence but exhibit a low incidence of breast tumors because their mammary glands are highly resistant to the influence; or, on the other hand, a strain may have susceptible mammary glands but develop few tumors because of the absence of the milk influence. Experiment 1 was designed to test for either possibility. Apparently, the mammary glands of mice of strains C57 black, I, and Y are relatively resistant to the milk influence since C_3H milk failed to evoke tumors in a high percentage of mice. Also, they do not possess the milk influence in any appreciable amount since their milk lowered the incidence of tumors in C_3H females. In strain C mice, however, the milk is also relatively free from the influence, but their mammary glands appear to be very susceptible to the action of the milk influence of C_3H mice. It may be postulated that strains C57 black, I, and Y are low-tumor strains because they lack both genetically susceptible mammary glands and the milk influence but that strain C is a low-tumor strain because it lacks the milk influence only.

Strain C mice are excellent experimental animals for an attack upon the problem of whether the high-tumor strains of mice are those possessing susceptible mammary glands which have been brought into contact with the milk influence. Experiments designed to show whether the milk influence of C_3H mice will transform strain C into a high-tumor line are now in progress. If these investigations reveal that strain C can be changed to a high-tumor strain, it would suggest that high-tumor lines of inbred mice are the result of a fortuitous exposure of susceptible mammary glands to an extrinsic agent and that this agent may be capable of self-reproduction. If the milk influence is active only in those strain C females which nurse C_3H females and their offspring do not develop breast tumors in subsequent generations, it will suggest that the milk influence is present or can propagate only in certain strains of mice. If the milk influence is a strain characteristic, it may have a genetic basis which would explain its occurrence only in high-tumor strains.

Bittner (*21*) stated that "females of low-tumor stocks nursed by high-tumor-strain mothers do not give a high tumor incidence. They may, however, transfer the milk influence to the young which they nurse * * * *". He also noted that breast tumors may arise in some high-tumor-strain mice which

> had the opportunity of nursing their mothers for from 1 to 24 hours before they were given to low-cancer-stock females * * * [and] * * * as the tumor incidence for the descendants of these cancerous fostered females decreased with each

generation, it is possible that the amount of the milk influence obtained by the females before they were fostered was too small for the active influence to be transferred to subsequent generations of their descendants.

In Bittner's experiments the mice of the subsequent generations were of the same strain as the mothers which supplied the small amount of milk influence. His observations indicate that the milk influence does not increase in amount in successive generations of susceptible mice, but fostered females of low-tumor stocks are able to transfer the milk influence to other animals. This suggests that a definite amount (threshold dose) of the milk influence is essential for the occurrence of breast tumors in mice. Strain C mice which have nursed C_3H females during the entire lactation period may be more suitable animals for this type of investigation than high-tumor females which may or may not have obtained a small amount of milk from their mothers.

Experiment 2, summarized in table 1, demonstrates clearly that hybrid females derived by mating C57 black females to strain I males were very susceptible to the extrachromosomal influence in the milk of female strain C_3H mice. The incidence of breast tumors in the fostered hybrids suggests that the hybrids were more susceptible than the inbred C57 black mice of experiment 1, but the findings are not conclusive for the following reasons: (1) As shown in table 1, the C57 black mice died at a relatively early average age; and (2) the mice included in the table as representing C57 black animals and C57 black $\times$ I hybrids were not offspring from the same C57 black mothers. This factor is taken into consideration in an experiment now in progress.

SUMMARY

The influence of foster nursing upon the occurrence of breast tumors in four strains of inbred mice and in one group of hybrids has been studied. Two experiments were performed. In one, mice from low-tumor strains C57 black, I, Y, and C were foster nursed by females of the high-tumor C_3H strain, and C_3H mice were fostered by females of the C, Y, and I strains. In the other, hybrid mice derived by mating C57 black females to strain I males were foster nursed by strain C_3H females. In both experiments unfostered litter mates served as controls.

Foster nursing of strain C_3H mice by females of strains C, Y, and I reduced the incidence of tumors in C_3H mice from 100 percent at an average age of 8.4 months to approximately 50 percent at an average age of about 12 months.

Foster nursing of mice of strains I, Y, and C57 black by females of strain C_3H increased the incidence of tumors in all three strains from 0 to approximately 15 percent.

Foster nursing of strain C mice by females of strain C_3H increased the incidence of tumors in strain C from 0 to 64 percent, at an average age of 8.1 months.

Foster nursing of C57 black $\times$ I hybrids by strain C_3H females increased the tumor incidence from 0 to 71 percent at an average age of 7.8 months.

A total of 65 breast-tumor-resistant mice were foster nursed by strain C_3H females and of these 26, or 40 percent, developed tumors, while of 70 nonfostered litter-mate controls, none developed a tumor.

The foster nursing of low-tumor-strain mice by high-tumor-strain females produced a definite increase in the incidence of breast tumors in the fostered mice.

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BREAST CANCER IN MICE AS INFLUENCED BY NURSING [1] [2]

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INTRODUCTION

Several theories have been proposed since the turn of the century to account for the etiology of breast cancer in mice. Some workers have held to the dominant theory, while others have stated that the evidence is in accord with the recessive hypothesis. The late Prof. A. S. Warthin, in a statement to his class in pathology in 1929, summarized the work completed to that date thus:

"Heredity may show itself in some cases as recessive, in others as dominant and in still others in a hit-or-miss fashion."

From a genetic standpoint many of the early experiments cannot be compared with present work because controlled material was not available nor was its value realized. The situation was expressed by Haldane (*1*) in an article entitled "The Genetics of Cancer":

> Besides the work described above, a good deal has been done on stock which was not genetically homogeneous. From this work it is clear that, while a tendency to spontaneous cancer is hereditary, it is not due to a single gene, dominant or recessive, * * *. But all work with genetically heterogeneous material is unsatisfactory, because any individual may die before reaching the cancer age, and no other individual will be of just the same genetical make-up. Hence really exact work is impossible.

In 1933 preliminary observations (*2*) were published on the breast-tumor ratios obtained in the first- and second-generation hybrids obtained by crossing individuals of high- and low-inbred tumor strains. The results secured in one cross were confirmed the next year by Korteweg (*3*), and reviewed in other works (*4–6*).

In every cross it was noted that if high-breast-tumor-stock females were mated to low-breast-tumor-stock males, the resulting female hybrid mice showed a breast-cancer incidence similar to that observed in the high-tumor-strain animals. When the hybrids were secured by making the reciprocal matings few tumors resulted in the first- and second-generation mice. (In this paper the terms "tumor" or "cancer" refer only to breast cancer in mice unless otherwise stated.) These data are represented in figure 1.

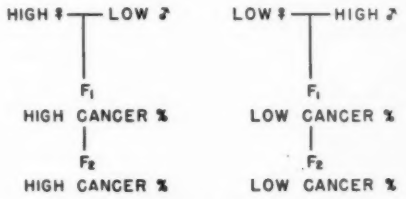

FIGURE 1.—Breast-tumor incidence obtained by reciprocal matings between high- and low-breast-tumor inbred lines of mice to demonstrate the maternal influence.

To explain these results it was evident that some influence, maternal or extra-chromosomal, was operative in the development of breast cancer in mice. As the maternal influence may be transferred only by the female parents in a cross, there are three possible methods of transmission to the progeny: (1) By means of the cytoplasm or cytoplasmic inheritance; (2) during intra-uterine development; and (3) by way of the mother's milk while nursing.

[1] *Supported by a grant-in-aid from the National Cancer Institute.*

[2] *George Chase Christian Lecture, University of Minnesota, Apr. 30, 1940.*

EXPERIMENTAL PROCEDURE

Early in 1934 young mice born to mothers of A high-breast-tumor stock were removed from their potentially cancerous mothers before they were 24 hours old and were given to females of low-breast-tumor stocks to be nursed (*6*, *7*). The low-tumor strains used were the CBA (X) and the C57 black (B). The tumor percentages observed in A stock virgin females, breeding females, and fostered females used as breeders are given in table 1.

TABLE 1.—*Breast-tumor percentages and average ages of virgin and breeding A stock females and fostered A stock females used as breeders*

A females	Mice	Mice developing cancer	Average age at death	
			Cancerous	Non-cancerous
	Number	*Percent*	*Months*	*Months*
Control, virgin	223	4.9	18.5	18.7
Control, breeding	376	95.7	10.1	11.9
Fostered by low-cancer stock ♂♀	127	7.9	11.2	17.7

The mammary-gland-cancer incidence obtained in virgin A stock females was 4.9 percent (*8*). The incidence for the breeding females of the sub-line used in these investigations was 95.7 percent. (This incidence is higher than that recorded in former articles describing this strain, as mice from all sub-lines have been included. The method of tabulation to be used in the future is, starting with the thirty-eighth inbred generation, to count only the first-generation progeny of the sisters of the female used to continue the stock. Thus, in any one generation all the animals tabulated are no further removed than cousins. This system has decreased greatly the number of individuals but should give a more homozygous sample of the strain. The last generation in which all of the representatives have been autopsied is the fifty-seventh.) The number of A stock females fostered by X or B stock mothers was 127, and they gave a cancer incidence of 7.9 percent (*9*).

Following this study and from observations obtained in hybrid animals, a hypothesis was proposed to account for the etiology of inherited breast cancer in mice (*5–10*). This theory assumes that at least three influences must be present and active before breast cancer will develop in high-tumor-strain animals and their hybrids. These are:

(1) A breast-cancer-producing influence which is normally obtained in the milk, while nursing, of a potentially cancerous female.

(2) A breast-cancer susceptibility which is transmitted by males and females of the high-tumor strain to their progeny. The evidence obtained to date is in accord with the theory that the susceptibility factor is a single dominant. This phase of the work is being investigated further as modifying factors may be involved.

(3) The growth of the mammary glands to a point where the cancerous change may take place as a result of the stimulation of the ovarian and associated hormones.

This theory is being presented at this time on the assumption that some of the experimental data to follow may be more clearly interpreted.

The relationship or the role played by the milk and/or the hormonal influences in the etiology of breast cancer in pure-stock animals may vary according to the strain. In the A strain they are probably of about equal importance, since without the production of young and the extensive growth of the mammary system few tumors result. Also, in fostered mice used as breeders the absence of the active milk influence greatly reduces the tumor incidence. In other strains as the D (*4*) and the C_3H stock described by Andervont

and McEleney (*11*) the incidence in virgin females is high.

Table 1 shows that the fostering of A stock females by low-tumor-stock mothers reduced the breast-cancer ratio. The data, which are presented graphically in figure 2 for the breeding stock used as controls and for those fostered by low-cancer stock, represent the proportion of the total to die cancerous or non-cancerous during each age period. Unless otherwise noted, the solid-black columns represent cancer. In addition to a lower breast-cancer incidence than the controls, the fostered A stock females lived longer than did the unfostered mice. The average ages at death of the non-cancerous mice of the two groups were 17.7 and 11.9 months, respectively.

The hormonal influence and genetic constitution of these groups of A stock breeding females were assumed to be the same. The only apparent difference was

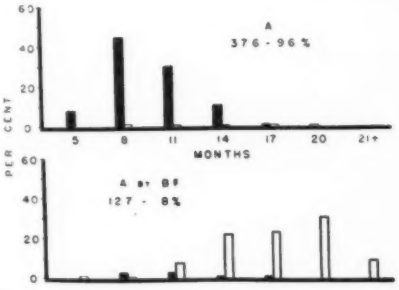

FIGURE 2.—The percentage of control A stock (A by A♀) and fostered A stock (A by B♀) to die cancerous or non-cancerous during each age period.

the milk obtained by the controls and the fostered animals.

A few of the A stock females which were fostered by low-tumor-stock females developed breast tumors. Their progeny and descendants are listed in table 2. Non-cancerous fostered females had 525 descendants continued, of which 6.1 percent developed tumors. The data are given graphically in figure 3. From table 2 it

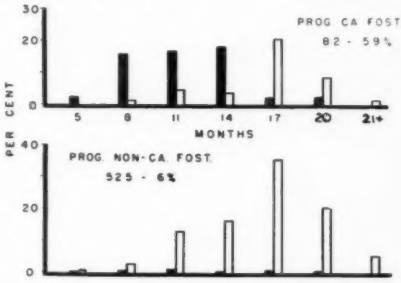

FIGURE 3.—The percentage of the progeny of cancerous and non-cancerous fostered A stock mice dying cancerous or non-cancerous each age period.

is evident that the non-cancerous young lived longer than did the mice which had growths. In table 3 are given for comparison the progeny of the litter mates (non-cancerous) of the fostered cancerous mice. These mothers have been separated into two groups, one group having progeny with a cancer incidence of 57.7 percent, and the other 4.1 percent of the total. Table 4 groups the descendants of the cancerous fostered and their non-cancerous litter mates having progeny with a high tumor rate, by the generation removed from the fostered animals. With each succeeding generation there is a decrease in the observed cancer incidence among the offspring for both types of fostered mothers.

TABLE 2.—*Breast-tumor incidence in the progeny of fostered A stock females which were breast cancerous and non-cancerous*

Fostered females used as mothers	Progeny	Mice developing cancer	Average age at death	
			Cancerous	Non-cancerous
	Number	*Percent*	*Months*	*Months*
Cancerous	82	58.5	11.8	17.2
Non-cancerous	525	6.1	12.6	16.8

TABLE 3.—*Breast-tumor incidence for progeny of fostered A stock females which died breast cancerous and for their litter mates which were non-cancerous*

A stock females used as maternal parent	Progeny	
	Number	Developed cancer
		Percent
Cancerous fostered A females	82	58.5
Non-cancerous females	26	57.7
Litter mates	169	4.1

Since the young of the A high-cancer-stock females had the opportunity of nursing their mothers for from 1 to 24 hours before they were given to low-cancer-stock females, it is possible that they obtained a sufficient amount of the active milk influence from their maternal parent to initiate the cancerous change, the other etiological influences being active. As the tumor incidence for the descendants of these cancerous fostered females decreased with each generation, it is possible that the amount of the milk influence obtained by the females before they were fostered was too small for the active influence to be transferred to subsequent generations of their descendants. The fostered mice which were non-cancerous but gave progeny with a considerable proportion cancerous all died before reaching the fourteenth month.

Fostered A stock mice which were non-cancerous but had cancerous progeny had 201 of their descendants mated. Of this number 5.5 percent developed tumors, a percentage corresponding to the incidence for the virgin A stock females (table 1). Their offspring which developed cancer had 19 of their young mated, and this group was 5.3 percent tumorous. The progeny of the 99 non-cancerous litter mates were 4.0 percent cancerous. Thus, if the descendant of a non-cancerous fostered female develops breast carcinoma, its descendants may show a low tumor incidence. In such cases the operation of an active milk influence is problematical. These tumors apparently are not transmitted and may result from a process analogous to somatic mutations. Changes in the somatic cells only are not inherited.

The progeny of 10 fostered A stock females which were non-tumorous were mated. The fostered females nursed 29 of their offspring and none developed tumors. Twenty-seven other young were given to high-cancer A stock mothers with a resulting cancer incidence of 88.9 percent. The increase in incidence was attributed to the transmission of the active milk influence by the potentially cancerous females.

TABLE 4.—*Cancer incidence of progeny, by generation, of cancerous A stock fostered females and of some non-cancerous litter mates*

Females used as mothers	Progeny by generation											
	First			Second			Third			Fourth		
	+[1]	−[2]	Cancer incidence	+[1]	−[2]	Cancer incidence	+[1]	−[2]	Cancer incidence	+[1]	−[2]	Cancer incidence
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*
Fostered cancerous	20	5	80	9	5	64	12	12	50	7	12	37
Fostered non-cancerous	12	8	60	3	3	50	0	0	.....	0	0	.....

[1] + = Developed cancer. [2] − = Did not develop cancer.

Fifteen mice were fostered from 1 to 5 days after birth. The incidence of cancer was 86.7 percent as compared with 7.9 percent for 127 mice fostered the day of birth (*12*). These observations were similar to those of Andervont and McEleney (*11*, *13*) for C_3H mice who also stated that the milk influence was present and constant in amount during the entire lactation period.

Hybrids were derived by reciprocal matings between representatives of the A high-tumor and the B low-tumor stocks. Figure 4 compares the A and B stock data

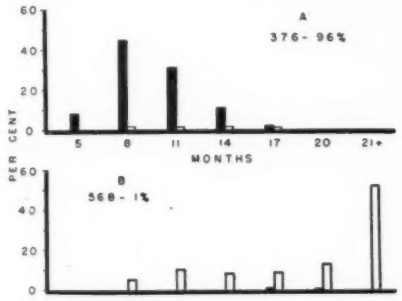

FIGURE 4.—The percentage of the A and B stock breeding females to die cancerous or noncancerous each age period.

giving the percentage of the total number cancerous (solid black) or nonbreast cancerous (open) observed during each age period. The B stock tumor data were from the publication of Little, Murray, and Cloudman (*14*).

Observations recorded for virgin hybrid females are given in table 5 with the average ages (*6*, *8*, *11*). As noted previously (table 1), A stock virgin female showed a low-breast-tumor incidence (4.9 percent), and similar observations were made on the hybrid females which were kept from producing young. Thus, A stock females and their hybrids must be used as breeders before breast carcinoma may be expected in any considerable percentage.

TABLE 5.—*Breast-tumor incidence and average ages for A and B stock mice and their hybrids kept as virgin females*

Virgin ♀ ♀	Total mice	Mice developing cancer		Average age at death	
				Cancerous	Noncancerous
	Number	*Number*	*Percent*	*Months*	*Months*
A	223	11	4.9	18.5	18.7
B [1]	133	0	.0	.....	26.4
ABF_1	111	5	4.5	21.9	26.7
BAF_1	104	0	.0	.....	28.6
ABF_2	119	2	1.7	20.0	23.1
BAF_2	120	0	.0	.....	24.6

[1] From Little, Murray, and Cloudman (*14*).

Table 6 gives the breast-tumor information for the A by B stock hybrids after they had been used as breeders. Each first generation had one group nursed by their maternal parent and another group nursed by the reciprocal stock female. In every case only one generation was fostered. For example, the BAF_1 by A♀ hybrids were made by mating B♀ × A♂, the young were removed from their B stock mothers and given to A strain females to be nursed. The F_2 progeny nursed their F_1 mothers, etc. The ABF_1 by B♀ mice were derived by mating the progeny of

TABLE 6.—*Breast tumor in breeding A, B, and reciprocal hybrid mice nursed by maternal parent or by females of the reciprocal stock*

Class	Number	Developed cancer	Average age at death		Living	Average age of living
			Cancer	Noncancerous		
		Percent	*Months*	*Months*	*Percent*	*Months*
A by A♀	376	95.7	10.1	11.9	0	.....
B by A♀	104	10.6	13.2	16.6	0	.....
ABF_1 by A♀	122	94.3	11.0	12.4	0	.....
BAF_1 by A♀	149	89.3	11.7	17.4	0	.....
ABF_2 by A♀	98	71.4	12.7	18.1	0	.....
Do	204	67.2	12.5	13.1	22.5	17.5
BAF_2 by A♀	123	39.1	12.3	12.9	53	13
B by B♀	568	.5	21.4	20.8	0	.....
A by B♀	127	7.9	11.2	17.7	0	.....
BAF_1 by B♀	118	1.7	12.5	21.9	0	.....
ABF_1 by B♀ [1]	162	.6	15.5	21.3	0	.....
BAF_2 by B♀	112	.0	.....	20.7	0	.....
Do	246	.0	.....	17.5	75	16.5
ABF_2 by B♀	149	.0	.....	16.3	72	18.4

[1] Progeny of A by B♀ mice mated to B stock males.

tested non-cancerous fostered A stock females (A by B♀) to B stock males. (Another group of ABF_1 by B♀ hybrids, made by mating high tumor A♀ to B♂, removing the young, and giving them to B stock females, will be mentioned later.)

The ABF_1 by A♀ breeding hybrid females were 94.3 percent cancerous and the ABF_1 by B♀ mice, 0.6 percent (table 6 and fig. 5). The average age at death of the

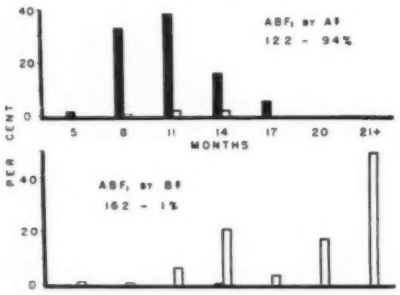

FIGURE 5.—The percentage of the ABF_1 by A♀ and the ABF_1 by B♀ hybrids to die cancerous or non-cancerous each age period.

non-cancerous mice of the latter class was 9 months later than the average age for similar mice of the ABF_1 by A♀ group. Figure 6 gives additional comparisons for these mice. When BAF_1 hybrids (B♀ × A♂) nursed their maternal parents, they (BAF_1 by B♀) were 1.7 percent cancerous, and after being fostered by A stock females (BAF_1 by A♀) they were 89.3 percent cancerous (fig. 7).

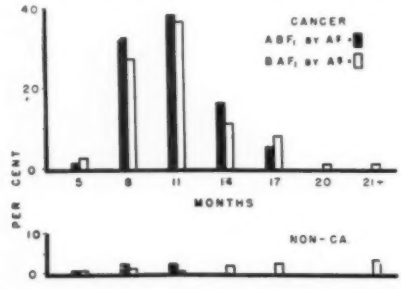

FIGURE 6.—The percentage of the ABF_1 by A♀ and the BAF_1 by A♀ hybrids to die cancerous or non-cancerous each age period.

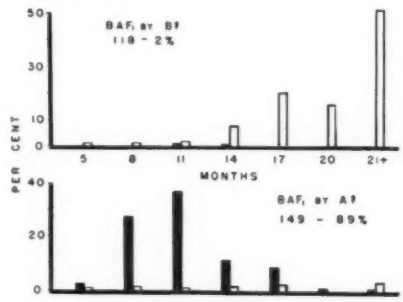

FIGURE 7.—The percentage of the BAF_1 by B♀ and the BAF_1 by A♀ hybrids to die cancerous or non-cancerous each age period.

The second generation data are illustrated in figures 8 and 9.

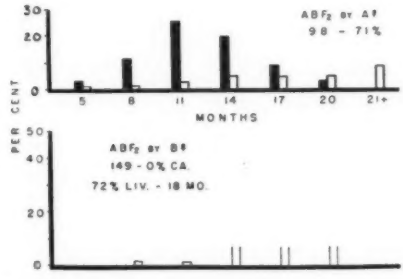

FIGURE 8.—The percentage of the ABF_2 by A♀ and the ABF_2 by B♀ hybrids to die cancerous or non-cancerous each age period.

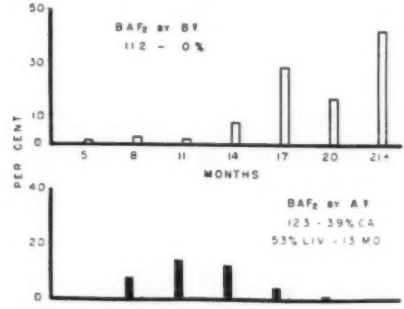

FIGURE 9.—The percentage of the BAF_2 by B♀ and the BAF_2 by A♀ hybrids to die cancerous or non-cancerous each age period.

To demonstrate the similarities and difference of the ABF_1 by A♀, and the ABF_2 by A♀ generation data the results are compared in figure 10. As will be noted

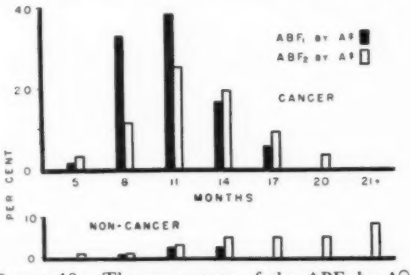

FIGURE 10.—The percentage of the ABF_1 by A♀ and the ABF_2 by A♀ hybrids to die cancerous or non-cancerous each age period.

the oldest non-cancerous first-generation animal died during the fourteenth monthly age period, an age attained by 23.6 percent of the F_2 generation non-cancerous animals. (Six percent of the non-cancerous BAF_1 by A♀ mice survived for a longer period than the average breast-tumor age.)

In addition to the hybrids obtained by the A × B strain crosses, a few first-generation animals were produced by mating males of the high-cancer Z (C_3H) strain (*6*) to B stock females. These mice were called the BZF_1 by B♀ hybrids when they nursed their maternal parents and BZF_1 by Z♀ mice after being fostered by Z stock females. The breast-tumor incidence for the group nursed by high-tumor Z stock mice was 79.1 percent, while that of the hybrids which remained with their maternal low-tumor-stock mothers was only 2.6 percent (table 7). The BZF_1 by Z♀ mice represent the same type of hybrids, having the same influences, as the BAF_1 by A♀ hybrids (removed from maternal low-tumor-stock mothers and fostered by high-tumor-stock females). While the breast-tumor incidence is not so high in the former cross (79.1 and 89.3 percent, respectively) the average age at death of the non-cancerous BZF_1 by Z♀ mice was 11.3 months as compared with 17.4 months for similar BAF_1 by A♀ animals (table 6). Tumors have not been recorded in Z by B♀ fostered females.

TABLE 7.—*Breast tumor in ♀ and B stock breeding females, B♀F_1 control and fostered hybrids, and fostered ♀ stock breeding females*

Stock of mice	Number	Cancer incidence	Average age at death	
			Cancerous	Non-cancerous
		Percent	*Months*	*Months*
Z	351	90.0	10.6	9.8
B	568	0.5	21.4	20.8
BZF_1 by B♀	39	2.6	7.5	18.5
BZF_1 by Z♀	43	79.1	11.3	11.4
Z by B♀	[1] 71	.0	.....	9.8

[1] Only animals over 6 months in age included; 42 percent of total living at average age of 14.5 months.

Table 8 gives tumor information for five groups of ABF_1 mice; the first two classes have been mentioned previously.

TABLE 8.—*Cancer incidence in ABF_1 hybrid breeding female mice according to the mothers which nursed them*

Class of hybrids	Nursed by—	Number	Cancer incidence	Average age at death	
				Cancerous	Non-cancerous
			Percent	*Months*	*Months*
ABF_1 by A♀	A♀	122	94.3	11.0	12.4
ABF_1 by B♀	A by B♀	162	.6	15.5	21.3
Do	B♀	37	8.1	17.6	20.2
ABF_1 by B♀ by BZF_1	B♀ by BZF_1 by B♀	48	4.2	12.5	23.4
ABF_1 by B♀ by BZF_1 by Z♀	B♀ by BZF_1 by Z♀	51	78.4	12.0	15.0

ABF_1 by A♀ hybrids (A♀ by B♂, nursed by high-cancer A♀) gave many cancers. After A stock females fostered by B♀ were mated to B stock males (A by B♀ by B♂), the resulting hybrids (ABF_1 by B♀) gave a low cancer incidence. When high-cancer A strain females were mated to B strain males and the young were fostered to B stock mothers, not many mice were observed with breast tumors. (These last two crosses are referred to as ABF_1 by B♀ hybrids. In the former the A stock females were fostered before they were mated to make the hybrids and only tested non-cancerous mothers were used; in the other the hybrids were fostered and, having high-cancer-stock mothers, had the opportunity of nursing from such females before they were placed with low-tumor-stock females. This may account for the observed difference in the breast-tumor ratios.)

The percentage of B stock females nursed by A strain mothers to develop tumors was 10.6 (table 6). Andervont and McEleney (*13*) reported an incidence of 9.1 percent when C_3H mice were used as foster mothers. Low-tumor-stock mice should not inherit the susceptibility to tumor; so when they are fostered by high-tumor females, few tumors should result. However, the fostered low-tumor mice do obtain the active milk influence when they suckle high-tumor females; and they can pass the influence on to other mice. This can be shown by foster nursing a low-tumor mouse by a high-tumor mouse. The low-tumor mouse is not susceptible, so it does not develop a tumor, but if it, in turn, is used to nurse susceptible mice, the susceptible mice will often develop tumor.

Another possible method by which the influence, normally obtained in the milk of high-tumor females while nursing, may be transferred is by means of the transplantation of tissues of grafts of organs of high-cancer-stock individuals which average 4 weeks in age (*15*). Spleen and thymus from young animals and lactating mammary tissue from mice which had had one litter were used. As all the grafts gave similar results, the observations are grouped in table 9 for comparison with the control BAF_1 and BAF_2 (by B♀) hybrids.

TABLE 9.—*Breast tumor in control and inoculated hybrid mice*

Stock	Nursed by—	Class	Number	Cancer incidence
				Percent
BAF_1	Low cancer stock ♀	Inoculated	46	21.7
Do	do	Control	108	1.9
BAF_2	do	Inoculated	11	18.2
Do	do	Control	112	0
Do	Inoculated BAF_1		17	29.4

The foregoing experiments were planned to determine the possible role played by the effects of nursing on inbred low- and high-tumor-stock mice and their hybrids, and the relationship between the milk and hormonal influences and the inherited breast-cancer-susceptibility factor. In another cross an attempt was made to maintain each influence as constant as possible with living material and to determine the tumor incidence in the hybrid-generation mice.

By taking the young away from high-cancer A stock females (95.7 percent cancerous) it is possible to lower this incidence by giving the progeny to low-cancer-stock females. In first-generation fostered mice the breast-cancer incidence was 7.9 percent (tables 1 and 6). By continuing the descendants of one fostered non-cancerous A stock female (No. 38432) a sub-line has been obtained which has given a breast tumor percentage of 3.6 for 192 mice representing 10 additional generations of inbreeding (table 10). This line of A

TABLE 10.—*Breast tumor in control (A) and fostered (A_x) A stock breeding female mice and their reciprocal hybrid generation mice*

Class	Number	Developed cancer	Average age at death		Living	Age of living
			Cancerous	Non-cancerous		
		Percent	*Months*	*Months*	*Percent*	*Months*
A	376	95.7	10.1	11.9		
A_x	192	3.6	11.5	17.4		
AA_xF_1 by A♀	41	87.8	9.0	7.0		
Do	73	17.8			82	2–9
AA_xF_2 by A♀	132	90.9	9.1	7.5	6	7–13
AA_xF_3 by A♀	93	92.5			6	10–11
Do	56	34.0			66	7–9
A_xAF_1 by A_x♀	108	0		15.5	19	14–19
					13	7–9
A_xAF_2 by A_x♀	148	.7	11.5	15.5	61	13–18

stock mice is referred to as the A_x strain (fostered by X or CBA females). Hybrids made by mating A high-tumor-strain females to A_x males were called the AA_xF_1 and the reciprocal cross (low tumor A_x♀×A♂) the A_xAF_1 generation.

The number of A_xAF_1 mice (low tumor ♀× high tumor♂) to be observed was 108, of which 68 percent died at an average age of 15.5 months. Of the remainder, 19.0 percent are living at ages ranging from 14 to 19 months and the others (13.0 percent) are from 7 to 9 months in age. The A_xAF_2 generation mice totaled 148 individuals. The surviving animals are from 13 to 18 months of age and make up 61.0 percent of the total. The average age of the non-cancerous mice that have

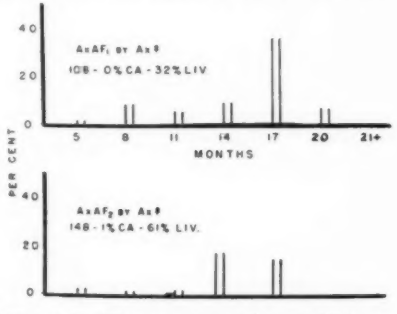

FIGURE 11.—The percentage of the A_xAF_1 by A_x ♀ and the A_xAF_2 by A_x ♀ hybrids to die cancerous or non-cancerous each age period.

died was 15.5 months. Tumors have been observed in none of the total A_xAF_1, and in only 0.7 percent of the A_xAF_2 population. The data are given in figure 11.

Two age groups of AA_xF_1 hybrids (high tumor ♀ × low tumor ♂) need to be considered. The older group has given a high tumor incidence. The AA_xF_2 generation is made up of 132 individuals with 6 percent of the total living between 7 and 13 months. The breast-tumor incidence for the total population is 90.9 percent, and the average tumor age 9.1 months. The first and second generation AA_x data are illustrated in figure 12. The AA_xF_3 population had been divided into two age classes; the older group of 93 mice has given a tumor incidence of 92.5 percent, with 6 percent of the total living. Figure 13 contrasts the cancer percentages for the inbred A stock females and the AA_xF_3 generation based on the older age group. Of significance here is that few of the non-cancerous mice lived to, or beyond the average cancer ages.

Figure 14 compares the percentage of the ABF_2 by A♀ and the AA_xF_2 by A♀ mice dying each age period from cancer or other causes. Of interest is the age at death of many of the non-cancerous ABF_2 by A♀ animals in that they survived beyond the average cancer age, 8 percent

of the total living over 21 months, an age attained by none of the cancerous individuals. In the ABF_1 by A♀ generation the non-cancerous mice all succumbed before reaching the older age periods (fig. 15), showing that they were probably genetically cancerous.

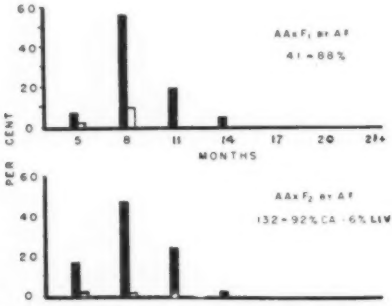

FIGURE 12.—The percentage of the AA_xF_1 by A♀ and the AA_xF_2 by A♀ hybrids to die cancerous or non-cancerous each age period.

The resemblance between the observations and the expected percentages of tumor formation according to the theory of breast-cancer etiology mentioned previously has been attempted by using a series of diagrams. These figures were designed to indicate the transmission of the milk influence by nursing, the genetic inheritance of the susceptibility constitution, and the hormonal condition. White areas in the figures imply that the potentiality of the influence is not sufficient, with few exceptions, to initiate that type of breast cancer which generally results from the combination of these influences. The fact that the area denoting the hormonal influence is not shaded does not signify that nulliparous females of the A strain do not secrete such hormones. It rather infers that, since the A stock virgin mice (and their hybrids) have a low breast-tumor incidence, the amount of the hormone present in these animals is either reduced to a minimum or that the amount needed for the cancerous change to take place is

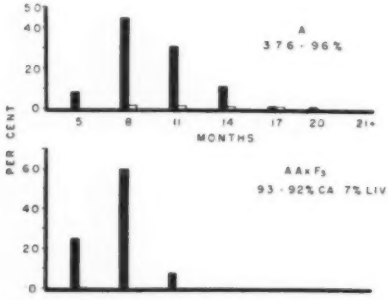

FIGURE 13.—The percentage of the A stock females and the AA_xF_1 hybrids to die cancerous or non-cancerous each age period.

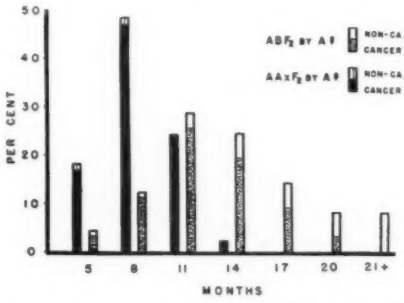

FIGURE 14.—The percentage of the ABF_2 by A♀ and the AA_xF_2 by A♀ hybrids to die cancerous or non-cancerous each age period.

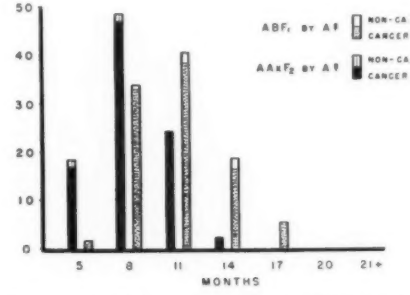

FIGURE 15.—The percentage of the ABF_1 by A♀ and the AA_xF_2 by A♀ hybrids to die cancerous or non-cancerous during each age period.

excessive when these animals are contrasted with virgin females of strains which have a high tumor incidence.

Figure 16 gives the "influence" constitution of a high-breast-cancerous breeding female (A stock) and a low-breast-cancerous breeding female (B stock). In the

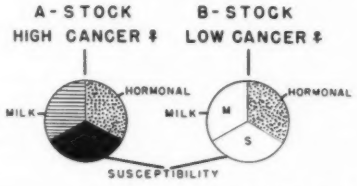

FIGURE 16.—Milk (M), hormonal (H), and susceptibility (S) influences of breast-tumor development in A (high-tumor) and B (low-tumor) stock breeding females. Breast tumor should theoretically result when all influences are active (shaded); but when one or more of the influences are inactive (unshaded), the mouse should be non-cancerous.

former all the influences would be active, and breast tumors generally would result. Females of a low-breast-tumor strain would have the hormonal state of breeding animals, but the milk influence would not

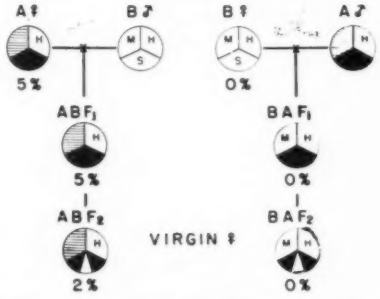

FIGURE 17.—Breast-tumor percentages observed in BA and AB virgin hybrid females.

be active and the mice could not transmit the genetic breast-cancer constitution.

By crossing A high-tumor and B low-tumor mice and maintaining the resulting F_1- and F_2- generation mice as virgins, few tumors would result, as shown in Figure 17. All these hybrid animals would have a deficient amount of the ovarian stimulation, and in addition the animals which nursed B stock females would receive only the inactive milk influence.

If the same cross were made and the hybrids used to produce progeny, the first-generation females nursed by A strain mothers might be expected to develop a maximum incidence of tumors (ABF_1 by

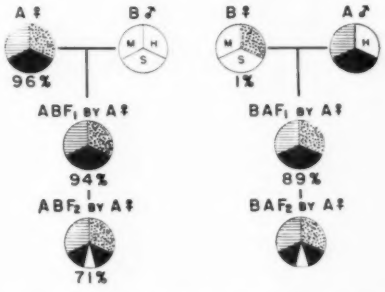

FIGURE 18.—Breast-tumor percentages observed in ABF_1, ABF_2, BAF_1, and BAF_2 hybrids, nursed by A♀.

A♀, = 94 percent, and BAF_1 by A♀, 89 percent; fig. 18). In the second-generation mice 25 percent of the total would be expected to be recessive for the dominant susceptibility factor. The recorded incidence of 17 percent for the ABF_2 by A♀ mice approached the expected ratio. Data for the BAF_2 by A♀ animals are not complete, as 53 percent of them are living; the tumor incidence thus far is 39.1 percent of the total.

Comparisons of the ABF_1 by B♀ with the BAF_1 by B♀ breeding females are indicated in figure 19. These animals would receive only the inactive milk influence from the B stock females; thus, none would be expected to develop tumors, and the mice observed showed 0.6 and 1.7 percent tumor incidence. No growths have been recorded in the second-generation animals

descended from the B stock nursed first-generation mice.

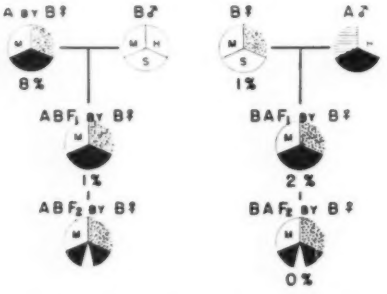

FIGURE 19.—Breast-tumor percentages observed in ABF_1, ABF_2, BAF_1, and BAF_2, nursed by B♀ hybrids.

Figure 20 contrasts the data for the ABF_1 and ABF_2 breeding and virgin females. The production of young with the increased ovarian activity and lactation associated with this phenomenon is the only apparent difference between the mice. The breast-tumor percentages secured for the parous and nulliparous animals are quite significant.

Expected and observed percentages for the cross between the low and high cancer A stock sub-lines are presented in figure 21. Hybrids having high-cancer-line mothers (AA_xF_1, F_2) gave high-tumor incidence in all generations (including the F_3, table 10) while mice descended from low-tumor A stock mothers developed few tumors (fig. 22).

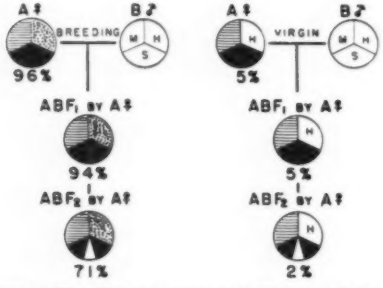

FIGURE 20.—Breast-tumor percentages observed in breeding and virgin ABF_1 and ABF_2 by A hybrid females.

Figure 23 demonstrates more clearly the operation of the breast-cancer-susceptibility constitution in the crosses under discussion. It represents the cross between the A stock females transmitting the susceptibility factor in the homozygous condition, and the B stock males, recessive for this influence. All first-generation mice would be heterozygous for the cancer complex, while F_2 mice, in which segregation of the susceptibility factor takes place, should give 3 cancerous: 1 non-cancerous individuals (75 percent expected, and 71 percent observed cancerous). Fostered A stock males and high-cancer A stock females would have, except for mutational changes, the same genetic constitution, i. e., cancerous. By mating such animals all the progeny should be homozygous and susceptible as far as the

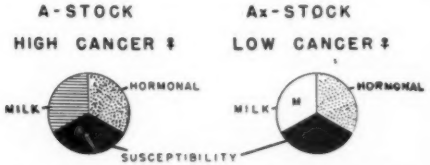

FIGURE 21.—Milk (M), hormonal (H), and susceptibility (S) influences of breast-tumor development in A (high-tumor) and A_x (low-tumor) stock breeding females.

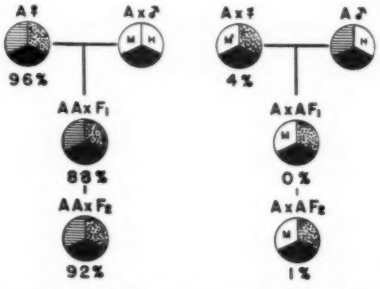

FIGURE 22.—Breast-tumor percentages observed in AA_xF_1 by A♀ and the A_xAF_1 by A_x♀ hybrids.

breast-cancer constitution is concerned.

In the presence of the other necessary influences of breast-cancer etiology, there should be little or no reduction in second-

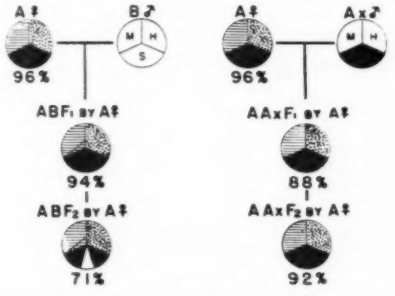

FIGURE 23.—Breast-tumor percentages observed in AB by A♀ and AA_x by A♀ hybrids.

generation mice (AA_xF_2 by A♀) as was observed for the ABF_2 by A♀ hybrids. That the result (90.9 percent, with 6 percent living) bears out the cancer homozygosity of the animals of this generation is apparent.

In this consideration of breast-cancer etiology in mice, we have assumed an exact correlation between the milk, hormonal, and susceptibility influences, and cancer development. For A stock breeding females this would indicate the development of spontaneous mammary carcinoma in every individual. That such is not the case is obvious. If these non-cancerous animals are mated and their progeny continued, the incidence of breast tumors among the offspring is very similar to the control stock, evidence that these mice are genetically cancerous and probably would develop the condition if they live longer. Breast tumors have also been recorded in a small percentage of animals where none were to be expected. By testing these animals it has been observed that there is no apparent increase of tumors in their progeny. This type of mammary carcinoma may have other contributing factors which have not been accounted for in the writer's hypothesis. Also to be considered in the development of these unusual tumors are physiological upsets in the equilibrium or relationship of the influences mentioned and changes characteristic of somatic mutations.

The injection of estrogenic hormones has failed to produce breast carcinoma in mice of the C57 black (B) stock (*16*). Twombly (*17*) observed, however, that if C57 black stock males which had been nursed by high-cancer R III stock females are treated with crystals of estrone, breast tumors resulted, but at a later age than they appeared in control R III males. R III males fostered by C57 black strain females gave a lower incidence of induced cancer than did the fostered B stock males.

Experimental work has thus demonstrated that an excessive dose of estrogenic hormone may produce carcinoma more easily in males of a strain nonsusceptible for breast cancer but having the milk influence active than in males of a susceptible strain which received the inactive milk influence while nursing.

Chemical analysis of the milk of high-cancer-stock lactating females is necessary to determine the characteristics of the breast-cancer-producing influence, work which is beyond the facilities of this laboratory. It will probably be found after this work is completed that some chemical agent—hormone, enzyme, or virus—will be the active principle involved. From the work on transplantation studies the causative agent may also be present in the normal tissues and organs of individuals of high-cancer strains of mice as well as in the milk.

CONCLUSIONS

The breast-tumor incidence normally obtained in females of high-tumor strains of mice may be reduced as the result of foster

nursing the young of such animals by low-tumor-stock females.

The maternal (extrachromosomal) influence of breast-cancer development in mice is transferred in the milk to the progeny while nursing.

The fostering of the progeny after they are 24 hours old has little effect on breast-tumor development.

Females of low-tumor stocks nursed by high-tumor-strain mothers do not give a high tumor incidence. They may, however, transfer the milk influence to the young which they nurse, and breast tumors may result in animals having all active etiological influences.

An influence similar to the milk influence may be present and active in other tissues and organs of cancerous-strain mice as indicated by data obtained from transplantation studies.

Breast-tumor ratios observed in hybrid animals were in accord with the genetic theory that breast-cancer susceptibility is transmitted as a single dominant factor.

Unfostered (high-tumor) and fostered (low-tumor) animals of an inbred high-tumor strain of mice are homozygous for the breast-cancer constitution.

Breast cancer in mice which is transmitted from generation to generation probably has a genetic basis as expressed by the inherited cancer susceptibility.

Three influences active in the etiology of inherited breast cancer in mice are (1) the milk influence, (2) inherited susceptibility, and (3) ovarian hormonal stimulation of the mammary gland.

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THE SIGNIFICANCE OF HORMONES IN THE ORIGIN OF CANCER [1]

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Since 1916 the study of the role of hormones in the production of cancer has been continuous, and constant progress has been made. However, this work has been made possible because of several previously established facts: (1) The incidence of spontaneous mammary cancer in mice—the species in which these investigations were carried out—varies greatly in different strains; (2) the hereditary cancer rate in successive generations remains constant (*1–28*); and (3) the cancer rate in breeding mice is higher than that in nonbreeding mice in almost all strains, but the degree of this difference differs in the various strains (*10, 11, 15, 29–31*).

Thus it was shown in 1916 (*32, 33*), and again in 1919 and the following years (*15, 31, 34–37*), that in strains with a high-cancer incidence the rate of mammary-gland carcinoma could be reduced to zero, or almost zero, if the ovaries were extirpated at the time of early sexual maturity. If the animals were spayed at the age of 5 to 6 months, the cancer rate was lowered, but not to such an extent as when the ovaries were removed earlier; and the cancer also appeared at a later date. If, however, the ovariectomy was performed at a still later date, the cancer rate rose still more; but the tumors appeared somewhat later, until in mice operated on at the age of about 10 months, the tumor incidence was the same as in normal mice.

It was already known at this time that the ovaries affect distant organs, such as the mammary glands, by means of hormones which they give off into the circulation (*38–43*). It was now shown in addition that through quantitatively graded withdrawal of ovarian hormones it was possible to grade quantitatively the incidence, the time of appearance, and the latent or preparatory period of mammary-gland carcinoma in mice and, experimentally, to make the behavior of the females of strains with a very high cancer incidence similar to that of males, in which mammary-gland carcinoma does not appear spontaneously. It was also possible to imitate experimentally the condition found in strains with various intermediate cancer rates and cancer ages.

Ancel and Bouin (*42*) attributed the proliferation of the mammary gland during the state of pseudopregnancy and pregnancy in the rabbit to the corpus luteum. In the guinea pig, the writer and Cora Hesselberg (*43*) observed a rhythmic growth of the mammary gland during the period of estrus under the influence of estrogen, whereas the corpus luteum did not exert a growth-stimulating effect on the mammary gland of this species, either during the sexual cycle or during the first stage of pregnancy. However, in the rabbit, rat, mouse, and cat, Turner and Frank (*44*) attributed the marked alveolar growth during pseudopregnancy and pregnancy to the combined action of estrogen and corpus luteum hormone (progestin).

Without entering here into a discussion as to the degree to which luteal hormone

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stimulates the growth of the mammary gland, there can be no doubt as to the effectiveness of estrogen; and it is, in the first place, through this hormone that the effect of the ovaries in the so-called spontaneous cancer is produced. We may then conclude that the spontaneous mammary-gland carcinoma of the mouse is due to the combined action of (1) factors transmitted by heredity, and (2) to the growth-promoting action of the estrogenic hormone, and that, given a constant quantity of the hereditary factor, the incidence of the spontaneous mammary-gland carcinoma can be quantitatively graded in accordance with the amount of estrogenic hormone given off by the ovary during the sexual cycle and during pregnancy. There is the possibility that during the latter condition other hormones may cooperate. Stated briefly: The so-called "spontaneous" mammary-gland carcinoma is a cancer produced by the estrogen given off rhythmically in mice hereditarily predisposed to this disease.

We may express these findings also in a somewhat different manner: The ovary discharges a certain amount of estrogen into the circulation at stated intervals, beginning spontaneously when the mouse is about 6 to 8 weeks old. If such discharges are brought to an end when the animal is 3 to 4 months old, mammary-gland carcinoma, as a rule, does not develop in any individual. If they cease in mice at the age of about 5 to 6 months, the carcinoma develops in a small number of mice following a prolonged preparatory period. Should estrogen continue to be released until the mice are 8 to 9 months old, the threshold has almost been reached, the incidence of cancer has now approximated a maximum for a given amount of estrogen and for a strain with a certain degree of hereditary predisposition. After a still longer period of discharge of this hormone, the percentage of tumors is not increased or the time of their appearance shortened. Under these conditions, carcinoma may appear a long time after the secretion of estrogen has been discontinued. It seems that, if a certain amount of stimulation has accumulated in a tissue, processes taking place elsewhere in the body are able to continue the work of the hormone, which, a considerable time after the action of hormones has ceased, may eventuate in cancer. But without this preceding period of stimulation, the second kind of stimuli, which may be of a less specific nature, would be unable to effect the cancerous transformation. Similar conditions seem to prevail in the production of cancer by means of carcinogenic substances. Bang (*45*) has observed that this type of cancer may develop a long time after the stimuli have ceased to act.

Quite recently Burns and Schenken (*46*) made an experiment that led to the same conclusions, although they applied a different procedure. Instead of using castrated females, they injected a certain quantity of estrogen at regular intervals into males belonging to a high-tumor strain. Injections continued for 1 month did not produce cancers; if continued for 2 to 3 month, tumors appeared, but with a diminished incidence as compared with that noted in breeding females. Injections over periods of 4 to 5 months yielded the maximum number of cancers; further increase in the length of time did not cause an additional increase in the tumor incidence. The results obtained in these two series of experiments are, in principle, identical; the differences are merely of a quantitative nature, owing to differences in the degree of hereditary predisposition in the strains used and in the amounts of estrogen given.

A corresponding relation between the quantity of cancer-producing substance

administered and the intensity of cancerigenic effect has been noted also in experiments with carcinogenic chemical substances. Oberling and the Guérins (*47*) have shown that the effects of benzpyrene injected subcutaneously into rats correspond to the quantities used. In this instance also, there is a certain amount of the stimulating substance which produces the maximum effect; and when this has been reached, a further increase in the amount given will not intensify the results. The cancerigenic action of a chemical substance other than a hormone corresponds, then, as far as these quantitative relations are concerned, to the action of hormones. However, the study of hormones as the cause of cancer preceded the study of these well-defined chemical carcinogenic substances.

The significance of hormones in the origin of cancer has so far been proven by examining the effects of graded diminutions in their amount as compared with that normally active in high-tumor strains. However, the effect of hormones on the development of tumors can be demonstrated not only by diminishing the amount of hormones, but also by increasing it over that given off by the ovaries under usual conditions. For this purpose, ovaries from female mice of high-cancer, partly inbred strains were transplanted into castrated brothers. This method was the reverse of one which we had used previously. But cancer did not develop in these male mice, in all probability because these experiments were not made on a sufficiently large scale. Nevertheless, as Murray and others have shown subsequently, positive results can be obtained in this way. We have resumed these investigations more recently and have likewise observed that mammary-gland carcinoma may develop in male mice into which ovaries have been transplanted, but we have found that tumors which seem to originate in the salivary gland may also appear under these conditions. Such tumors, which somewhat resemble sarcoma but are not connective-tissue growths, have been seen so far only in two normal control male mice of strain A, but five such tumors have already been found in a relatively small group of castrated males implanted with ovaries. Further investigations must show whether this experimental interference increases the incidence of these peculiar new formations.[2]

Cori (*37*) attempted to produce mammary-gland carcinoma in ovariectomized mice by the injection of estrogenic hormones. His results were negative, presumably because the quantities of the hormone used were too small. Somewhat later Lacassagne (*48–54*) succeeded in producing mammary-gland carcinoma in male mice belonging to high-tumor strains by injecting large quantities of estrogen over long periods of time. Lacassagne's experiments, although few in number, demonstrated that it is possible to produce mammary-gland carcinoma in high-tumor-strain male mice through injection of very large amounts of estrogen. Negative results were obtained when estrogen was administered to male mice belonging to strains in which carcinoma of the mammary gland does not, or only very rarely, occur spontaneously, and in which therefore the amount of estrogen normally released into the circulation by the ovaries is likewise ineffective; for even additional amounts of estrogen introduced parenterally were insufficient. Lacassagne's experiments give further proof that carcinoma of the mammary gland does not occur in male mice of high-tumor strains be-

[2] *Quite recently I learned that Dr. Stewart and Dr. Grady of the National Cancer Institute have likewise found these tumors in males of the A and some other strains. In this case the tumors evidently arose spontaneously.*

cause the hormone necessary for the stimulation of the mammary gland is lacking. These experiments were confirmed and extended by Burrows (*55*) (1935), Gardner (*56*) (1937), and by Gardner, Smith, Allen, and Strong (*57*) (1936).

More recently (1939) carcinoma of the mammary gland has been produced in mice, in which it otherwise would not have appeared, in still another way, namely, by transplantation of lobes of the anterior hypophysis under conditions which made possible the long-continued, and probably permanent, survival of the transplants (*58*). By transferring the glands from several brothers to a sister in a closely inbred strain, the growth and secretion of the mammary gland were much accelerated and intensified, and the percentage of mammary-gland carcinoma was noticeably increased. The carcinomas appeared from about 5 or 6 to 10 months after transplantation of the hypophyseal lobes. However, these transplants were effective only if ovaries were present in the hosts; there was no definite effect in ovariectomized females or in males. Cancers were produced only in strains of mice in which there was a strong tendency to the development of spontaneous mammary-gland carcinoma.

A difference was noted in the reaction of mice belonging to strains with a low mammary-gland-carcinoma incidence on the one hand, and in ovariectomized females or males belonging to high-tumor strains on the other. While in neither of these two strains did mammary-gland carcinoma develop, the mammary gland remained inactive, or almost inactive, in the males and spayed females in contrast with conditions found in the low-tumor strains, in which the mammary gland was stimulated to further proliferation and secretion. But this increased growth activity of various portions of the mammary gland in such strains did not reach the point where transformation to cancer could take place; and at an intermediate stage the growth ceased.

If the ovaries are needed for the effectiveness of the hypophyseal grafts, a simple explanation would be that the transplanted pituitary lobes stimulate the ovarian follicles to increased estrogen production, which would then act on the mammary gland. However, according to Turner and his associates (*59*, *60*) estrogen does not act directly on the mammary gland but affects it only indirectly by way of the anterior lobe of the hypophysis, which under the influence of estrogen is induced to give off a specific hormone, mammogen, the latter being responsible for the growth of the mammary gland. If we accept this interpretation, we would have to conclude that the ovaries are necessary for the full effectiveness of the transplants, because they stimulate the latter to secrete mammogen.

As to the manner in which hormones call forth the production of cancer, several significant facts have been established, especially in regard to the mammary gland. In the first place, a sudden change does not occur from the resting gland, or from the gland undergoing the ordinary cyclic-growth processes, either during the sexual cycle or during pregnancy, or lactation, into carcinoma under the influence of an estrogenic substance, although such an effect might be conceivable. Instead, there is a gradually increasing stimulation of the mammary gland which may express itself either in mitotic proliferation or in secretion, or more generally in a combination of these two processes. This gland does not respond very readily to the action of estrogen; it seems to offer a strong resistance which can be overcome only with considerable effort. Only slowly do active growth and secretion processes set

in, and then they progress gradually. As a rule, different parts of the gland may be affected at the same time, although one part may show a somewhat more advanced stage than another; but they all are pushed along to proliferation step by step, and, ultimately, the last stage is reached in this developmental process and the growth becomes abnormal.

When, as a result of very active growth, the small ducts and acini have been pressed together rather closely, or when the effect in separate small tubules has become very intense, they may begin to coil round in an abnormal manner. This may occur in acini which still possess normal structure and in which the size of the nucleus and cytoplasm are still normal, or it may be preceded by abnormalities in nuclei, some of which may be large while others have the usual size; there may appear a single prominent nucleolus-like structure in the center of the nucleus. However, real giant cells are not seen under these conditions. Preceded thus by intense growth and secretion processes which may be considered precancerous, the cancerous transformation takes place. Structurally no sharp distinction between these different phases can be made.

Abnormal glandular structures begin to develop which may protrude as papillae, or penetrate actively into the surrounding stroma and continue this growth even if the injection of estrogen has been diminished. In this way cancer is produced first in one place; and somewhat later, in another, the same stage may be reached. The number of areas thus affected varies in different cases. There may be only one place which is markedly pushed along in this growth process, but very often different areas are active at the same time. It seems that in the so-called spontaneous carcinoma, and in other instances when the amount of active hormone substance is not excessive, the tendency of the cancerous transformation to proceed in single areas is greater than is found experimentally when large quantities of estrogenic substance are injected over long periods of time.

The end ducts and the acini developing from them, rather than the individual cells, are the structural units of the mammary gland which are affected by these stimuli and which respond with growth and secretion processes. In some cases, however, growth processes may start around a large duct, from which smaller ducts and acini may grow out which soon become carcinomatous. It is especially in such places that keratinization and pearl formation may be noticeable. Evidently the collecting ducts have still retained the tendency of the epidermis to form squamous epithelium and to keratinize. The estrogenic hormones thus produce cancer of the mammary gland by stimulating the gland tissue to greater and greater proliferation, until finally the threshold of normal growth processes has been exceeded, and the gland then passes into the phase of carcinomatous growth. This may be associated with only a slight decrease in the normal organization of the mammary gland and in the normal tissue interaction between parenchyma and stroma, or there may be a furthergoing disorganization. This is, however, a change which is not specific for cancerous growth; it may be observed, although more moderately, also in regenerative processes, for instance, in the epidermis and in the connective tissue during the process of wound healing. It is the result of the stimulated condition of the tissues which may lead to excessive and abnormal movements, as well as to an increased size of cells, and a more rapid and abnormal cell multiplication.

Whenever a hormone is the stimulus which induces carcinomatous growth in

the parencyhma of a given organ, we should expect the cancerous change to affect the whole organ diffusely. To some extent this condition is realized in mammary-gland carcinoma in mice, inasmuch as there is here a tendency to multicentric growth and to a gradually increasing involvement of the different portions of the gland in the cancerous growth. But this diffuse growth is not complete; there is usually a differential growth response in parts of this organ, which must be due to the cooperation of accessory variable factors. Some of these factors are known; thus, the condition of the fat tissue and the connective tissue stroma which influence growth processes may, perhaps, differ in different areas of the mammary gland in mice; but it is probable that there are in addition other differences as yet undetermined.

Accessory factors seem to participate in the origin of carcinoma of the vagina and cervix of the mouse. In general, whenever a substance—whether a hormone or an ordinary carcinogenic compound—affects a tissue diffusely and cancer results, there should take place a general, or at least a multicentric, transformation of normal into cancerous tissue. Such a condition may be approximated in the mammary gland, the vagina, the cervix, and also in the liver of rats fed with O-amino-azotoluol (Sasaki and Yoshida (*61*), and others). In the case of the liver, by way of multiple nodular hyperplasia and adenoma, ultimately a malignant hepatoma is produced, that affects the whole organ. Likewise in the thyroid gland, and perhaps also in the prostate, multiple adenomata may become transformed into carcinoma.

We see, then, that the administration of large doses of estrogen gradually causes the development of small tubules and acini, some of which undergo a moderate degree of hypertrophy, associated in some areas with mitotic proliferation, and in others with marked secretory processes. Turner (*62*) has found in various species of rodents that under the influence of theelin it is almost exclusively the duct system which is developed; and also in the growth of the mammary gland which takes place in castrated male mice after transplantation of ovaries, it has been noted that it is the ducts which proliferate, while acinar or alveolar growth is lacking. Turner (*62*) and also Gardner (*63*) consider the acinar growth to be associated with a diminished growth of the ducts, which is found in experiments with long-continued injections of estradiol in mice as a pathological phenomenon. We would consider it probable that this difference in the mode of growth of the mammary gland under different conditions is due to different degrees of stimulation. Whenever there is a marked stimulation of the gland, a relatively rapid transformation of ducts into acini occurs; while a milder degree of stimulation affects mainly the duct system, and acinus formation is much retarded.

According to our interpretation, hormones would then produce cancer by stimulating epithelial structures in such a way that, step by step, the growth becomes intensified, until the cancerous transformation occurs. Other interpretations have been given as to the manner in which hormones produce cancer. It has been suggested that the hormones act by increasing the quantity of mammary-gland tissue, that the resulting decrease in the number of available gland cells facilitates the occurrence of somatic mutations in previously normal cells, and that such mutations presumably are also caused by the action of estrogen. However, it is very improbable that this is the essential condition underlying carcinoma formation for the following reasons: (1) Mammary-gland carcinoma can form, espe-

cially in cases in which the stimulation is not very intense, from a relatively small area in the gland without a preceding new formation of a large amount of gland tissue; (2) the mammary gland of pregnancy in which a large amount of growing gland tissue is available, is not known to pass directly into the cancerous state; and (3) in the case of experimental cancer of vagina and cervix in the mouse, the same factors are active as in cancer of the mammary gland, and in the former the amount of additional tissue produced in which the cancerous transformation takes place, is very slight. Evidently the cancerous transformation does not depend upon the number of cells newly produced. During pregnancy a rapid proliferation of the whole gland takes place, which proceeds in an orderly manner and which terminates in an equally rapid retrogression. The cancerous change depends, rather, upon the intensification of growth activity which continues over a relatively long period of time and advances, step by step, until the end stage is reached. This is common to the various organs in which cancer can be produced by hormones or by other chemical-physical means.

Lacassagne (*64*) and others have suggested that the development of cancer under the influence of estrogen follows the cystic dilatation of ducts in which colostrum, or milk containing estrogen, is retained. The estrogen thus has a chance to act on the duct tissue and to exert its carcinogenic effects. However, the formation of cysts containing secretion products is not characteristic of glands in which carcinoma originates. Moreover, in the large majority of cases mammary-gland cancer begins in the smallest ducts or acini in which secretion or stagnation of secreted fluid is entirely lacking, and lastly, all the evidence points to the conclusion that estrogenic substances are distinct from the typical carcinogenic substances. The latter may or may not be estrogenic, and estrogenic substances as a rule are not carcinogenic. In confirmation of this conclusion which we previously expressed, Robson and Bonser (*65*) noted that triphenylethylene may produce carcinoma of the mammary gland, and Lacassagne (*66*) found that stilboestrol likewise is effective in this respect. These substances differ in their structure and action from the typical carcinogenic substances, but they are estrogenic.

It has also been suggested by Lacassagne (*67*) that estrogen may cause a primary stimulation of the mammary gland, but that it is the hereditary factor which in a subsequent phase brings about the actual transformation of the growing tissue into cancer. However, as stated previously, the relation between stimulating and hereditary factors is just the reverse; the hereditary factor acts first and makes it possible for the stimulating factor to effect the cancerous transformation in a subsequent phase. With this interpretation the findings of Gardner, Smith, Allen, and Strong (*68*, *69*) are essentially in agreement, although they attribute the cancerigenic effect of estrogen to the feminizing action of this substance. "Feminization" is a general term which in this instance designates a stimulating action not only on the mammary gland but also on other sex organs, such as the vagina and uterus, and which includes the mental corollaries associated with these changes. But it is solely the action of the estrogenic substances on the mammary gland which is of significance in the production of mammary-gland carcinoma.

It has already been mentioned that transplantation of anterior pituitary glands which survive in the new host is a very efficient way for producing mammary-

gland carcinoma. While an excess of growth-producing hormones is not directly administered in this case this result is accomplished in an indirect manner. There are some indications that also the extirpation of the greater part of the ovaries in the guinea pig may induce some precancerous changes under certain conditions. Lipschütz (*70*) observed in such animals a glandular hypertrophy of the endometrium and in one case noted a precancerous condition of the cervix. He attributed these changes to the mediation of the anterior pituitary gland. Bagg (*71*) found that he effected a stimulation of the breast tissue in mice and rats by forced breeding, when the course of one pregnancy was followed quickly by the next one without interruption by a period of nursing, and, accordingly, one growth period of the mammary gland was followed directly by another. There can be little doubt that the stimulation in this instance was caused by hormones active during pregnancy, which exceeded the stimulation produced under normal conditions in the same length of time. It is not certain, however, how far estrogenic or luteal hormones were concerned in producing this effect. Furthermore, it is not certain or even very probable that mechanical injury caused by stagnation of milk in the larger ducts induces regenerative growth. According to Fekete (*72*), the omission of periods of nursing is not an essential factor in this procedure; it seems rather that in these experiments an increase in the number of pregnancies was responsible for the increase in the number of mammary-gland carcinomas. This would be in agreement with our earlier observations concerning the enhancing effect of breeding on the incidence of these tumors. Such a mode of increasing the number of mammary-gland carcinomas resembles the other methods used for the production of mammary-gland carcinoma, and there is no reason for attributing to it a specific action. As stated previously, the ordinary so-called spontaneous cancers of the mammary gland in mice are likewise essentially "hormone" cancers.

The facts so far established concerning the differences in the incidence of mammary-gland carcinoma in different strains of mice make it almost certain that all degrees of susceptibility to the cancerigenic action of hormones may be found in different strains. There is probably an absolute zero point at which not even the most intensive stimulation will be effective, and there is another point at which the normally acting dose of estrogen is as yet almost, although not entirely ineffective, but in which a stronger dose of estrogen experimentally administered increases the number of individuals which will be affected by cancer. There is, then, an ascending scale in the percentage of female mice in which mammary-gland carcinoma develops in the various strains under the influence of spontaneously produced and secreted estrogen, and in which the cancer rate can be increased the more, the greater the amount of injected estrogen; correspondingly, the the carcinoma occurs at an earlier age.

The effect of the experimentally increased amount of estrogen is readily demonstrable if we choose for these experiments virgin mice of strains in which the normal mammary-gland-cancer incidence is low and that of breeding mice is high. An end point is conceivable at which even in virgin mice the normal cancer rate is so great and cancer appears so early, that experimental administration of an increased quantity of estrogen is ineffective in further increasing the rate and rapidity of appearance. While in males the spontaneous appearance of mammary-gland carcinoma is extremely rare, it is possible to produce it in them through injection of large doses of

estrogen at approximately the same rate as in females of the same strains.

In some cases Bagg (*73*) called forth mammary adenocarcinoma in rats by means of intensive breeding, while in other rats adenoma and adenofibroma of the mammary gland originated under these conditions. This is in accordance with the occasional spontaneous occurrence of benign tumors without the aid of experimentally applied stimuli. In a certain strain of rats Geschickter (*74*) succeeded, by the use of various estrogenic substances, among them stilboestrol, in producing adenocarcinoma in a considerable number. These experiments are of additional interest because Geschickter could show that there was a proportionality between the dose of estrogen administered and the time needed for the development of cancer, an observation which corresponds to our findings in mice spayed at various periods of life, and to the experiments of Burns and Schenken (*46*) to which reference has already been made. Furthermore, in the rats the hormone exerted a diffuse effect on the whole mammary-gland tissue. This result would be expected, in accordance with the fact that estrogenic hormone affects all of the mammary-gland tissue. The first change in these experiments indicating a cancerous transformation consisted in a coiling of the smallest ducts similar to that which had been observed as a frequent initial change in the mammary gland of mice. There remains the possibility that cancer was produced in many of these rats because they had a certain strain predisposition to the development of mammary-gland carcinoma, which became manifest as the result of an intense stimulation of this organ. However, the spontaneous occurrence of mammary carcinoma—that is, of carcinoma developing under the influence of a normal amount of estrogenic hormone given off under normal conditions—had not been observed in this strain of rats.

We have so far studied largely the mammary-gland carcinoma of the mouse and to a less extent that of the rat. It is natural that the analysis of the origin of tumors in general has been concerned largely with a few typical tumors, characteristic of certain species and organs, which occur with great frequency and which are therefore readily accessible for study. To this class belong the mammary-gland carcinoma of the mouse, the sarcoma of the rat, and the fowl sarcoma; but in addition to these malignant tumors, there is found a benign type of tumor, the adenofibroma in the mammary gland of the rat, which is also a relatively common occurrence. We have studied this type of tumor at various times, beginning in 1901 (*75*), mainly by means of transplantation. Loeb, Heiman (*76*, *77*), and Emge (*78–83*) have shown that the success of grafting this tumor is determined in the first place by the individuality differentials of host and transplant, and also by hormones, in particular ovarian, and pituitary or pituitary-like hormones. However, according to Heiman,[3] reactions against hormones differ in tumors transplanted into female and male rats in accordance with the variations in the two sexes in regard to the spontaneous action of hormones. Heiman has further shown that testosterone inhibits the growth of the transplanted tumor, and that a larger amount of testosterone is needed to cause inhibition in young than is required in older rats. From the point of view of the origin of tumors, the finding is of interest that while the fibromatous connective-tissue part of the tumor may in some instances be transformed into sarcoma by serial transplantation (Heiman and Emge)—the change which, according to Heiman, may be promoted by the action

[3] *Heiman, J.: Personal communication.*

of certain hormones—it has not been possible so far to transform the adenomatous constituent into carcinoma through administration of hormones, which are able to change normal mammary-gland tissue into carcinoma or adenocarcinoma, not only in the mouse but also in the rat. However, only a few attempts have been made so far to analyze the origin of this tumor.

Bagg observed the appearance of adenocarcinoma in some rats subjected to intensive breeding, but in certain instances instead of adenocarcinoma, adenofibromata developed in the mammary gland. While under normal conditions carcinoma of the mammary gland in rats does not occur at all, or only very rarely, the adenofibroma is much more common. In these experiments the frequency of these types of tumors was therefore reversed. It is probable that hereditary factors may play a certain role. Geschickter (*74*) used a certain inbred strain of rats, and it appears very likely that an inherited strain characteristic was responsible for the results obtained.

The recent investigations of Wolfe, Burack, and Wright (*84*) are of interest in this connection. They observed a relatively frequent occurrence of adenofibroma in a certain inbred strain of rats; in some animals also fibroma or adenocarcinoma developed. This strain of rats was characterized by abnormalities in the sexual cycle, by a deficiency in ovulation, and a great prominence of the interstitial gland. These authors seem inclined, therefore, to connect the frequent occurrence of the benign tumors in this strain with these abnormalities in the sexual cycle, and in particular with the lengthening of the estrus period which was associated in some instances with an increased action of estrogen, and perhaps also with increased activity of the anterior hypophysis. However, these differences in the action of hormones were essentially statistical, and it seems doubtful whether they can explain the tumor formation in individual animals. It is possible that special inherited strain characteristics may play a role in the origin of these tumors. How far hormones also are concerned in this process, as the direct stimuli acting on the mammary gland tissue, needs further investigation.

While so far chiefly the hormonal factor in the etiology of mammary gland carcinoma of the mouse has been emphasized, it must not be forgotten that the hormonal factor is active only in combination with a hereditary factor, that this latter factor differs greatly in different strains of mice, and that it is transmitted from generation to generation. Although in the present paper the action of hormones in the development of cancer has been considered first, the effect of heredity studied earlier yielded the first important results. About 40 years ago, observations were made by various investigators concerning the incidence of cancer in certain strains of animals. In cattle and rats we noted that the so-called endemic occurrence of cancer was due to a hereditary familial condition. We then began to study with Miss Lathrop, in Granby, the incidence of cancer in various strains of mice and found very great differences, which remained constant in successive generations. At about the same time Tyzzer (*5–7*) attempted to establish the significance of the hereditary factor, by comparing the incidence of cancer of the lung in the offspring of mice in which such pulmonary tumors had occurred, with that of the offspring of mice in which they had not occurred. These studies were later extended, and in the course of our investigations we observed that in hybridization between strains markedly differing in their cancer rate, the mother

strain preponderated in determining the tumor incidence of the offspring.

Murray and Little (*85, 86*), who made similar observations, attributed the preponderance of the mother strain to extrachromosomal factors; and Bittner (*87*) found that the milk of the mother transmitted to the children a factor which helped to determine the cancer rate of the hybrids. However, according to Murray and Little (*88*), after serial backcrossing of various kinds through eight generations, the cancer rate becomes uniformly low, without regard to the gene constitution of the offspring or the presence or lack of the milk factor.

Whatever the nature of the hereditary factor may be, there can be no doubt that different strains of mice differ greatly in the hereditarily transmitted tendency to mammary-gland carcinoma, and that this difference in the hereditary-cancer rate can readily be established, provided the strength of the stimulating factor is the same in the different strains.

The question arose as to wherein these different degrees of predisposition to cancer consist. The possibility had to be considered that predisposition to cancer was due to inherited differences in the amount of estrogenic hormone produced and given off into the circulation in different strains of mice. However, Loeb and Genther (*89*) did not find any correlation between cancer incidence and characteristics of the sexual cycle in these strains, although inherited differences in the length of the sexual cycle exist in certain strains, as had been found already by Allen (*90*). Lacassagne (*50, 52*) and Hardé (*91*) believed they had established such a connection between the characteristics of the sexual cycle and the mammary-gland-tumor rate.

Investigations in this problem were resumed (Loeb et al. (*92, 94*)) because we felt that the differences in the cancer incidence of the strains used previously by us had not been sufficiently definite. In these later studies it could be demonstrated again that no correlation exists between the characteristics of the sexual cycle and the incidence of mammary-gland carcinoma. Bonser (*95*) arrived at the same conclusion. However, inasmuch as certain differences in the character of the sexual cycle may be found in different strains and the amount of estrogen given off in each sexual cycle may differ in different strains, it is probable that the incidence of cancer and also the cancer age do not exactly denote the hereditarily transmitted tendency to mammary-gland carcinoma. Yet it may be safely assumed that the observed cancer incidence and cancer age at least approximately indicate the strength of the hereditary tendency to the development of cancer. This conclusion is supported by the fact that the readiness with which injections of measured quantities of estrogen produce mammary-gland cancers in male mice belonging to different strains, approximately parallels the normal incidence of cancer in the females belonging to these strains.

A second possibility which might explain the differences in the tendency to develop mammary-gland carcinoma in different strains is that these are due to differences in the readiness with which estrogenic hormones are destroyed or eliminated in the injected animal. But if this were the true cause of these hereditary strain differences, one would expect that in various strains cancers caused by estrogenic hormones in other parts of the body would take a parallel course to the cancer incidence in the mammary gland. This is, however, not the case.

There is one condition which corresponds approximately to the hereditary predis-

position in different strains, namely, the readiness with which the normal mammary-gland tissue reacts to stimulation by estrogenic hormones with normal growth processes. In a general way, it may be stated that the greater the hereditary tendency to mammary-gland carcinoma, the more readily will the stimulation by a certain quantity of hormone applied over a period of time lead to the formation of branching ducts, of small collections of acini, of lobules, of secreting and proliferating ducts and acini, and gradually to the development of precancerous and cancerous tissue (*34*, *36*, *50*, *52*, *58*, *67–69*, *96–98*). There is no sudden transition from hitherto quiescent ducts or acini to cancerous tissues; it is necessary for these structural units first to pass through the normal activities, and only when the possible limit of their normal growth has been reached will the change to cancerous growth occur. It is this difference in the responsiveness of the mammary-gland tissue which essentially represents the mechanism causing the great difference in the cancer incidence in different strains (*34*, *36*, *50*, *52*, *58*, *67–69*, *96–98*). A strain possessing a strong tendency to the development of mammary-gland cancer shows a reaction to a relatively small amount of estrogen similar to that which a strain with a lesser hereditary tendency shows to a larger quantity of estrogen. An added amount of stimulation by estrogen can compensate for a certain difference in hereditary predisposition.

In the case of carcinoma of the mammary gland there is an inverse ratio between the amount of stimulation and of hereditary factor needed. The smaller the amount of hereditary factor in a certain mouse, the greater is the amount of stimulating factor that has to be applied if cancer is to develop, and conversely, the greater the amount of hereditary tendency to mammary gland carcinoma, the smaller is the amount of stimulating factor needed. Without the application of stimulating factors, the transformation of normal into cancerous tissue does not take place in the mammary gland, even if the degree of hereditary tendency is very great, and a very intense application of stimuli may lead to the development of cancer even in some individuals which belong to a strain possessing a very low degree of hereditary tendency, though the ability of stimuli in this respect is, also, not absolute but limited. In a general way, this relationship between hereditary tendency and stimulating factors may be expressed by the equation $S \times H = C$,[4] although this equation is probably not accurate in a quantitative sense. There are indications that similar relations prevail also in other types of cancer in which the relative importance of hereditary and stimulating factors varies greatly.

According to the findings of Bittner (*87*), it would in addition be necessary to assume that the ability of different strains to transmit to the offspring with the milk a mammary-gland-cancer-producing factor also bears some relation to the hereditary tendency to produce cancer. However, an increase in the strength of the stimuli applied to the mammary gland can overcome a lack of this mammary-gland-cancer-producing factor under certain conditions; thus in hybrids, in which the mother strain had a low cancer incidence, the lack of a tendency to produce mammary-gland carcinoma can be overcome by increasing the intensity of normal stimulation (Bagg (*71*)). Moreover, this ability to transmit the tendency to acquire mammary-gland carcinoma by a factor contained in the milk of the mother is apparently limited to the mammary gland of the mouse. So far,

[4] *S, signifies stimulating factor; H, hereditary factor; and C, the resulting cancer.*

it has not been found when the cancer originated in organs other than the mammary gland.

Essentially the hereditary factor in cancer consists in an inherited degree of responsiveness of the mammary gland to stimulation. In certain closely inbred strains this responsiveness is very great, while in others it is slight or almost lacking. This holds good in general, although there are indications that in addition certain strain peculiarities may perhaps exist. Notwithstanding the fact that the individuality differentials of different mice in such inbred strains become almost identical, great differences are found in the number of individuals which actually become affected by cancer of the mammary gland in different strains; and this number and the average age at which cancer appears remain approximately constant in successive generations. One would expect that the different strains differ merely in the amount of stimulation necessary, or in the time required for eliciting the development of carcinoma in all the individuals of the same strain. Given a constant intensity of stimulation applied in a unit of time, cancer should appear at about the same age in all the individuals of a certain strain. The fact that this is not the case indicates that other variable factors complicate these relations, but these factors are largely unknown as yet. Notwithstanding these individual differences within the same strain, the degree of hereditary tendency in different individuals in the same strain seems to be the same, as is indicated by the fact that individuals which belong to the same strain and which are affected by cancer, transmit the same amount of hereditary predisposition to their offspring as do individuals which are not affected by cancer.

The variable factors of a nonhereditary character which may complicate the action of the two main factors are not altogether unknown. One of these factors consists in the effects of undernourishment on the mammary gland of mice. If on account of disease or deficient diet, a mouse begins to lose weight, atrophy takes place in the fat tissue of the breast and at the same time the relative or absolute amount of hyaline connective tissue increases. As a result of such changes, the mammary gland ducts and acini situated in the small vacuolated or collapsed fat tissue undergo atrophy and after some time disappear entirely. Likewise, the hyaline connective tissue may injure the ducts severely by exerting pressure on them and by interfering with the supply of foodstuffs which they need. The conditions relating to the state of the fat tissue and the amount of the hyaline connective tissue vary greatly in different individuals in accordance with the state of nourishment. A variable factor is present which may favor or inhibit the growth of the mammary gland and influence also the growth of cancer. These observations are in agreement with, and help to explain the experiments of Tannenbaum (*99*) concerning the effect of underfeeding on the incidence of tumors.

A given degree of hereditary tendency to the development of carcinoma is specific not for an individual as a whole, but only for certain organs and tissues of the individual. Thus the degree of hereditary tendency as expressed in percentage of incidence of mammary-gland carcinoma in mice, applies only to carcinoma of the mammary gland but does not apply to carcinoma of the cervix or vagina, to cancer of the skin, or to sarcoma, which is the cancer of the connective tissue. In two different strains, mammary-gland carcinoma may show very great differences in frequency, whereas carcinoma of the cervix and vagina may be equally frequent in

both, and the reverse condition may also occur (*100, 101*). The hereditary tendency to cancer is, therefore, an inherited organ peculiarity, corresponding to the many other organ and tissue peculiarities which a more thorough comparison of the life history of individuals belonging to different strains reveals. The responsiveness of the mammary-gland tissue to stimuli has so far been tested almost exclusively by means of hormones. As to whether there exist, also, in different strains corresponding differences in their responsiveness to other stimuli, no definite data are at hand. It is conceivable, for instance, that the relative responsiveness of the mammary-gland tissue to chemical carcinogenic stimuli in different strains may differ from that exhibited when hormones are used.

Estrogenic hormones not only call forth the development of carcinoma of the mammary gland in mice, but also they may induce carcinoma of the cervix. We have referred already to this type of experimental carcinoma as well as to the observation of Lipschütz (*70*), who observed a precancerous change in a guinea pig in which a great part of the ovary had been removed. Overholser and Allen (*102, 103*) attempted the production of carcinoma of the cervix in eight monkeys, to which they administered a combination of estrogen and lutein hormone, or estrogen alone. In all but one of these, repeated mechanical traumatisms were added to the hormone action. In one monkey they succeeded in producing a precancerous condition, as squamous cell nests invaded the underlying connective tissue. Perry and Ginzton (*104, 105*) applied a combination of 1:2:5:6-dibenzanthracene and estrogen to mice and noted the development of precancerous changes.

In our experiments, as well as in those of Lacassagne (*106*) and Gardner, Allen, Smith, and Strong (*107*), abnormal proliferative changes were elicited in the cervix merely through subcutaneous injections of estrogen. We observed the development of carcinoma which invaded the underlying stroma over wide areas and penetrated into blood vessels. Gardner, Allen, Smith, and Strong provided an additional proof of the cancerous nature of these proliferations by transplanting them successfully into other individuals of the same strain. We found, moreover, that it is not only possible to produce carcinoma or precancerous conditions in the cervix, but also that corresponding changes may be induced over wide areas in the vagina. In our experiments the latter changes were at least as frequent as the proliferative changes in the cervix, evidently because the epithelium of the cervix and that of the vagina are very similar, and only quantitative differences exist as to the manner in which each responds to the estrogenic and luteal hormones.

While there are graded differences in the response of the estrogenic and luteal hormones in the different parts of the sex tract in the direction from vagina to uterus (*108*), in vagina and lower cervix the behavior of the epithelial tissues differs only slightly, and correspondingly, the carcinomatous reactions are almost the same in these areas. The nearer we approach the uterus, the less is the tendency of the epithelial tissue to respond to estrogenic hormone and the greater is the tendency to the reduction of the squamous epithelium, and in the end to the production of cylindrical epithelium. This characteristic feature appears not only in the normal, but also in the carcinomatous growth (*100, 101*). In a transitional zone the carcinomatous epithelium can produce squamous epithelial cell nests as well as cylindrical strands, and these structures may

alternate. In the uterus proper the epithelium loses to a great extent the potentiality to the formation of squamous-cell epithelium. If, under pathological conditions, the uterine cavity of the mouse is lined with squamous epithelium, this change is due, at least in the large majority of cases, not to a real metaplasia of the uterine epithelium, but to an ingrowth of squamous epithelium of the cervix into the uterus; and from the surface this epithelium may grow downward into many gland ducts (*109*).

In numerous instances it is possible to observe at the upper end of the cervix and at the beginning of the uterus, a struggle between these two types of lining epithelium, and very often it is possible to note the invasion and replacement of the cylindrical by the squamous epithelium. This substitution may extend over wide areas, even without the previous occurrence of an infection and ulceration in the uterine cavity. The vaginal and cervical epithelium proves to be the stronger type because it has the greater proliferative power. For this reason it may proceed readily to the production of squamous epithelium and to the production of precancerous and cancerous tissue; but the precancerous tissue consists here of squamous epithelium, not because the squamous epithelium as such has a peculiar ability to produce cancerous tissue, but because the greater potentiality to growth in the vaginal and cervical epithelial tissue manifests itself in the greater tendency in a first stage to form squamous epithelium, and having passed through this stage, to become transformed into carcinomatous tissue. The squamous epithelium represents a transitional condition between the resting epithelium and the cancerous tissue. The same factor that induces the formation of squamous epithelium leads ultimately to the production of cancer.

The uterine epithelium in the mouse is much less responsive to the action of growth-stimulating estrogenic hormones. It is very difficult, or perhaps impossible, to transform it into typical keratinized squamous epithelium, and we found it equally impossible by the application of hormones to produce a carcinoma in the uterus proper of the mouse. Occasionally there was a downward growth of normal glands through the muscle layers of the uterus, but no real precancerous condition developed (*109–111*). However, this does not mean that under no conditions could such a change occur. We have to deal in this and many other instances only with quantitative differences in growth energy of certain tissues, and there may be variations in this respect, also, in different species. Thus in the human species carcinoma may develop in the fundus of the uterus, although even here this change occurs more rarely than in the cervix.

The formation or lack of formation of cancerous changes depends upon quantitative differences in tissue response, and these differences may apply to individuals, strains, and species. In the latter they are, as a rule, greater than between different strains of the same species, and these species differences apply to tissue reactions in general. Thus, in the guinea pig a corpus luteum hormone induces mainly growth processes in the stroma of the uterine mucosa (*39, 112–114*), whereas in the rabbit it induces largely certain metabolic and structural differentiations, although growth processes take place in this species in the uterine epithelium (*38, 115*). It is this hereditary difference in responsiveness to stimuli in different species which determines also to some extent the efficacy of the stimuli in the production of cancer. In addition, differences may exist in the nature of the stimuli which are active in different species. Thus in women, cancer of the

cervix is more frequent than cancer of the vagina, because in the former apparently other stimuli besides hormones are active, namely, inflammatory and mechanical irritations. In the mouse, the latter are much less significant, although we have found some indications that here also they may play a minor role in the production of precancerous and cancerous changes.

A very definite example of species differences in the responsiveness of tissues to tumor-producing stimuli is seen in the behavior of connective tissue and unstriated muscle tissue of the uterus in mouse and guinea pig. Large doses of estrogen produce in the mouse a diffuse deposit of a hyaline substance in mucosa and muscularis, which does not result in tumorlike formations (*116*). In the guinea pig, however, Nelson (*117*) has found that administration of this substance leads to fibromyomatous tumors, and Lipschütz and his coworkers (*118–121*), as well as Moricard and Cauchoix (*122*) have shown that fibrous nodules develop also in various abdominal organs. We shall refer to these tumors shortly again. After cessation of the injections, the hyaline deposited in the uterus of the mouse is replaced by ordinary connective tissue and the fibromyomatous nodules in the guinea pig disappear (*123*). A real irreversible change characteristic of cancerous growth has, therefore, not yet taken place in these fibroids.

Other marked examples of such species differences become manifest also if we compare cancer in mammary gland, cervix, and uterus, and some other tumors, of mouse, rat, and guinea pig. In the mouse, the incidence of mammary-gland carcinoma in various strains differs greatly, but in a mixed population the incidence would presumably be intermediate between the rate noted in high- and low-tumor strains; while the types of adenofibroma which are relatively frequent in the rat, seem to be unknown, or at least very rare, in the mouse. On the other hand, a real mammary-gland carcinoma is found only very seldom in the rat. In the guinea pig, tumors of the mammary gland are very uncommon. Spontaneous tumors, if they occur at all, are very rare in the cervix, vagina, and uterus in all three of these species. However, it is possible, through the application of estrogenic hormones, to produce fibromyomatous nodules in the uterus of the guinea pig.

Witherspoon (*124, 125*), in 1933, suggested that fibroids in women may be caused by the action of estrogenic hormones. Nelson (*117*) produced experimentally subperitoneal fibromata in the guinea pig by injecting estrogen over a number of months; and some muscle fibers were admixed to the fibromatous growth. Lipschütz and his collaborators (*118–121*), as well as Moricard and Cauchoix (*122*), noted under the same conditions in the guinea pig, the development also of extrauterine fibrous tumors in various abdominal organs and in the mesentery. According to Lipschütz, these reach a larger size in female than in male animals, and they develop more readily in castrated than in normal females. Moricard and Cauchoix (*122*) observed that such tumors form readily around a foreign body introduced into the uterus. Under the influence of estrogen the connective-tissue reaction which normally takes place around foreign bodies evidently is much intensified and leads to tumorlike new formation.

As to the interpretation of the spreading of the estrogen effect to the connective tissue of various areas in the abdominal cavity, it appears most probable that this is due to the fact that the estrogen injected subcutaneously is not limited in its distribution to the uterus proper, but is deposited and becomes active also in adjoining areas, where it causes a stimulation of the

fibroblasts and, perhaps, of the cells of the unstriated muscle tissue. The corpus luteum hormone also affects the connective tissue of the sex tract in the guinea pig; it induces a slight direct stimulation or a marked sensitization of these cells. The sphere of action of the luteal hormone is more limited and specific, comprising only the uterine cervix and the uterus proper; furthermore, it is only the mucosa, and not the subperitoneal tissue, which is responsive to this hormone (*39*, *112–114*). These differences between the specificity of the estrogen and luteal effects are of great interest.

The conditions under which fibromyomatous nodules are produced in the guinea pig indicate a certain lack of specificity in the action of estrogenic hormones; but there are observations which prove still further absence of specificity in the case of experimentally produced sarcoma. A number of investigators have noted the development of spindle-cell sarcoma in mice injected with estrogenic susbtances in oil (Cori (*37*); Gardner, Smith, Strong, and Allen; Lacassagne; Loeb, Suntzeff, and Burns (*109*)). In the large majority of these cases the sarcoma originated at the site of the injection; but in one instance we observed that it developed at a distance from the place of injection, in the vaginal tissue, and Lacassagne (*127*) noted its occurrence in the bladder. We also found that long-continued injection of other substances, such as luteal hormone and liver extract, have the same effect as estrogen, and more recently we obtained sarcomata in four mice injected with a solution of HCl buffered with phthalein (*128*, *128a*). Nishiyama (*129*) and Takizawa (*130*) also produced these tumors in rats and mice in places where concentrated solutions of various sugars had been injected over long periods of time. It is not only the connective-tissue cells which can thus be stimulated to undergo a sarcomatous transformation; striated muscle tissue also reacted in this way in the animals observed by us. These data indicate that sarcoma develops under conditions much less specific than carcinoma. As we have seen, the epithelium of various secondary sex organs, on account of its chemical constitution, is adapted in a specific manner to a certain hormone, which is able to induce growth processes and thus ultimately may cause the formation of carcinoma. On the other hand, connective-tissue cells, and perhaps also striated muscle tissue, respond to a much wider range of substances with growth processes, and in the end with a sarcomatous transformation, but these substances lack the high degree of specificity which the hormones possess. It is possible that even purely mechanical stimuli may, in some cases, be able to accomplish the development of a sarcoma, but apparently other growth-inducing factors are more effective.

In this instance, as well as in the others considered in this connection, we have evidently to deal with differences in the mode of reaction of different tissues. Such differences are apparently of a quantitative rather than of a qualitative kind, because it is known that epithelial surfaces as well may undergo a cancerous transformation under the influence of long-continued stimulation of various kinds.

Lymphoid tumors originating in the thymus or lymph glands have also been observed not only in mice injected with estrogen (Gardner (*56*), Lacassagne (*131*), Gardner, Smith, Strong, and Allen (*132*), Gardner, Kirshbaum, and Strong (*133*)), but also in mice treated with carcinogenic chemicals or exposed to Roentgen rays (Furth (*134*, *135*)). Likewise, lymphatic leukemia may develop under these conditions. In these cases distant actions of

estrogen are involved in which the degree of specificity is slight. Wherein the cancer-producing influence consists here, whether in a direct stimulation of the tissues by the agents mentioned, or in regenerative or compensatory processes, is not known.

We have given reasons for assuming that it is the continued effect of growth stimuli and the intensity of the growth reactions resulting from these stimulations that eventuate in the cancerous transformation. However, it is possible that there is a certain phase of the growth process which is especially prone to lead to the cancerous change, and that this phase may be more accentuated in certain kinds of growth stimulation than in others. We have seen, furthermore, in the case of mammary-gland cancer that the degree of responsiveness of the tissues to growth stimuli corresponds to the strength of the hereditary tendency of different strains of mice to acquire this type of carcinoma; that the hereditary predisposition is a characteristic of the individual organs and tissues rather than of the individual as a whole; that the type of cancerous growth which results from such stimulations differs in accordance with the physiological and structural characteristics of the tissue from which the tumor is derived; and finally, that it is possible for certain hormones to provide the effective growth stimuli. In the kinds of cancer which have been considered in this paper, one particular hormone, namely, estrogen, caused the cancerous transformation, and estrogen was presumably concerned in the production of mammary-gland carcinoma which was induced by transplantation of hypophyseal tissue.

While the estrogenic and hypophyseal hormones may primarily induce growth processes and carcinoma formation in the mammary gland it is well known that various glands with internal secretions interact with one another, and it is therefore conceivable that, secondarily, they may also be concerned in this process. In the mouse long-continued injection of estrogen may lead to marked increase in size of the anterior lobe of the hypophysis, but the degree varies greatly in different instances; in some cases it may exceed the limits of mere hypertrophy or hyperplasia and may assume the character of an adenomatous growth.

Cramer and Horning (*136–138*) noted a correspondence between the degree of enlargement of the pituitary and the strength of the hereditary tendency to the development of mammary-gland carcinoma in various strains. They suggested that variations in the activity of the hypophysis are responsible for the differences in the readiness with which cancer develops in these strains. In our experiments, a strain in which the resistance to the cancerous transformation is very great showed the most pronounced enlargement of the pituitary under the influence of estrogen injections. While our experiments show that an excess of anterior hypophyseal tissue may augment the stimulating factors to such an extent that the incidence of mammary gland carcinoma increases, there is no indication that the hypophysis is the seat of the hereditary predisposition.

Cramer and Horning (*139*) attribute great significance also to the cortex of the adrenal gland, in the production of the cancerous change whereas the medulla of the adrenal gland is believed to inhibit this process. This conclusion is based on a comparison of the conditions found in various strains of mice. In those in which the incidence was very high, a brown degeneration was found frequently in the medulla, and as a result of these degenerative changes the inhibiting effect of the medullary cells seemed to have been destroyed (*139*). However, such a parallelism between

these medullary changes and cancer incidence does not hold good generally, and it is lacking in the strains most commonly used in the study of cancer in mice. Certain observations suggest that the adrenal cortex may substitute for the ovary in initiating the cancerous formation of the mammary gland. In some mice, signs of estrus may appear in vagina and uterus, although the ovaries have been completely extirpated some time previously (Burns, Suntzeff, and Loeb (*128*); Perry (*140*)). There is a possibility that the adrenal cortex may supply the necessary estrogenic stimuli in such animals, and this assumption is supported by recent experiments of Woolley and Little (*141*). They found that if mice are ovariectomized as early as 24 hours after birth, a marked development of the mammary gland takes place in the course of time, and in older mice hypertrophy of the uterus may be noted. Typical estrus cycles occurred in some cases, and about 27 percent of all the animals developed mammary-gland carcinoma. Woolley and Little further observed that the adrenal cortex had undergone nodular hyperplasia, which in three mice gave origin to malignant tumors. This combined occurrence of growth processes in the mammary gland and of hypertrophy or hyperplasia in the adrenal cortex suggests that the proliferative changes in the sex organs may have been caused by the production of estrogenic hormones in the adrenal cortex. It seems, therefore, that ovariectomy in newborn mice may lead to secondary changes in the adrenal cortex which thus assumes certain functions ordinarily exerted by the ovaries.

It may be concluded that estrogenic hormones produce cancerous changes in mice in which the responsiveness to growth stimuli on the part of a tissue has passed a certain limit. When this cancerous change has occurred, the estrogenic hormone—and the same is presumably true also of other hormones—is no longer needed. The cancerous growth continues, but the changes which have taken place in the normal tissue during this cancerous transformation at the same time abolish the responsiveness to hormones which normal tissues possess. On the other hand, adenomatous tissue may still possess such a responsiveness to hormones, as we observed in the course of transplantation of adenomas of the mammary gland in rats.

That hormones may induce cancer formation has been proved only as far as estrogenic hormones and anterior pituitary transplants are concerned. Still, it is not probable that these effects are limited to the sex hormones. The estrogenic hormones are effective for two reasons: (1) They induce growth processes in the receptive organs, namely, mammary gland, vagina, and cervix—the requirement which primarily has to be fulfilled if a hormone is to elicit cancer formation; and (2) estrogen does not induce a refractory state to such a marked degree as do certain other hormones, especially those that are protein in nature.

The prolonged administration of the hormone must not lead to the development of a refractory state in the animal to which the hormone is being administered, or in the tissue on which the hormone acts, inasmuch as such a condition would prevent continued growth stimulation. Such a refractory state may be due to either of two conditions: (1) The development of immune substances which inactivate the hormone after long-continued injections. These immune substances have been called antihormones by Collip; they may form especially if the hormones are protein in nature; (2) refractory states may develop also under conditions which seem to preclude the formation of immune substances or anti-

hormones, following an interval of stimulation. Thus, for instance, application of inorganic iodine salts in the guinea pig in a first period causes a stimulation of the thyroid gland, while in a succeeding period this effect is replaced by a refractory state (Rabinowitch (*142*)). Similarly, Blumenthal and the writer (in as yet unpublished experiments) observed that after injections of the corpus luteum hormone, which stimulates the mucosa of the uterus of the guinea pig, there may be a period in which the stimulating effect decreases. On the other hand, it seems that rhythmic growth stimulations, which hormones exert under normal conditions, are not followed by refractory states. In fact, as far as is known at present, the estrogenic hormones do not under normal conditions elicit a refractory state. As to the pituitary transplants, which also induce the formation of carcinoma, the character of the hormone which is effective is not definitely known.

In our early papers on the cancerigenic function of ovarian hormones we suggested the possibility that other hormones also may be able to induce cancer formation. In general, we stated that whenever a hormone takes part in initiating and in maintaining growth processes, it may also be a factor, either direct or auxiliary, in the production of cancer. In this connection it must be remembered that hormones may affect not only the tissues on which they act directly, but also other tissues which, as far as their proliferative activities are concerned, are correlated in some way with these primary tissues. Thus the changes in the sexual cycle in the guinea pig, which are caused by the action of hormones, concern not only the secondary sex organs, but also organs far distant from uterus, vagina, and mammary gland, such as the epidermis (*143*). In this way hormones may indirectly affect the development of cancerous growth in distant organs, and in addition they play a significant role in the origin of benign tumors, for instance, the fibromata of the uterus. There is some evidence that prostatic hypertrophy may be caused by the action of hormones, although the mode of action in this condition is not as yet quite certain.

The cooperation of hormones has been suggested, also, in the experimental teratoma of the testis in birds. It is possible to produce teratoma in the testis of birds by injection of zinc salts (Michalowsky). There is a seasonal factor in the occurrence of these tumors, since they are obtained in the spring months at a time when spermatogenesis begins in the fowl. Falin (*144, 145*) believes that they originate from the primary germ cells, the gonocytes, and he assumes that gonadotropic hormones stimulate the gonocytes to develop into teratomatous structures. Champy and Lavedan (*146*) produced seminomas in the testicle of the fowl by partial extirpation of this gland. In the regenerating gland spermatogonia, instead of becoming transformed into spermatocytes and spermatozoa, failed to undergo the normal differentiation; instead, they proliferated and gave origin to these primitive tumors. These investigators attributed the abnormal growth at least partly to an excessive secretion of gonadotropic hormone by the hypophysis, which had been stimulated by the partial castration. These are instances in which the role of hormones in the production of tumors or tumorlike growths has not actually been proven, but the suggestion that hormones may be concerned in such processes seems reasonable.

We have assumed so far that continued stimulation in association with hereditary factors accomplishes the cancerous transformation. It may furthermore be assumed that each tissue constituent of an organ possesses a certain threshold of stim-

ulation and that if this threshold is exceeded, the normal equilibrium of this tissue is lost, and a permanent cancerous transformation takes place, which is irreversible. There are two alternative interpretations which have in common the belief that the two causative conditions referred to, namely, the presence of stimulating and hereditary factors, alone are not able to produce the cancerous change, but that they merely prepare the soil for another factor, which accomplishes the actual production of cancer. Such a function has been attributed to somatic mutations, constituting random effects, which are thought to occur the more readily, the larger the number of cells exposed to such an effect. We have pointed out that the sequence of tissue changes which take place under the influence of hormone stimulation makes this interpretation very improbable.

According to Rous (*147*, *148*), and Andrewes (*149*) the stimulated state of a tissue induced by hormones prepares this tissue for the invasion of a virus, which is the direct cause of the cancerous transformation. It has been shown, first by Rous (*147*, *148*), and subsequently by Lucké (*150*, *151*), that certain cancerous growths are induced by viruses. But the problem as to whether viruses play this important role in all kinds of cancers, or as to whether tissues may undergo a cancerous transformation directly without the intervention of viruses needs further analysis. It is an important problem, not only because of its bearing on the etiology of tumors but also because of its general biological significance. If viruses accompany all cancer cells and spur them to constant proliferation, an extraneous factor would furnish the stimulus for the cancerous growth; otherwise we would have to attribute to normal tissue cells the potential power to produce autocatalytically, through alterations of their normal metabolism, a substance which causes these intensified growth processes and associated phenomena indefinitely.

However this may be, there is still a third condition which should be fulfilled if cancer is to develop under the influence of hormones. The hormones, and this applies also to all other growth-producing factors, must not induce strictly cyclic reactions. Many of the growth processes which take place normally in the organism are rhythmic, or at least cyclic, in character; they are caused by factors or stimuli originating within or without the organism, which likewise are rhythmic or cyclic. In some cases the stimulus may be continuous; yet the reaction becomes cyclic, because the tissue which functions as substratum changes in such a way that the stimulus ceases to be effective and the tissue becomes refractory to the stimulus and therefore returns to its original condition. It may be stated that, in general, those growth reactions which lead to cancer formation must not be completely rhythmic or cyclic in character. Successive stimuli push the tissue into a new and different condition, and additional stimuli add to this deviation from the normal. Hormones which lead to cancer formation must cease to initiate completely rhythmic or cyclic proliferative changes. If the hormones continue to be produced or discharged cyclically, the response on the part of the tissue, because of a change in this tissue, must cease to be entirely rhythmic or cyclic if cancer is to develop. Gradually the tissue changes from the normal to the cancerous state which, in contrast to the normal labile tissue equilibrium, is stable and unalterable. Under various pathological conditions, whether caused by simple physical-chemical alterations in the environment or by infectious processes, the tissues may respond to stim-

uli with noncyclical growth processes. Changes in the substratum which occur in old age may have similar effects, and such conditions may then also be factors in cancer formation. The cancerous transformation may be reached by way of regenerative, hypertrophic, and hyperplastic processes, and by conditions designated as benign tumors. All these may function as stages transitional to the cancerous phase.

If there is a rhythmic discharge of ovarian hormones, and presumably also of pituitary as well as pituitary-like hormones, during the normal sexual activity of the animal and during pregnancy, the response of the tissue cannot be completely rhythmic, and the state of the tissue at the end of the cycle is not exactly the same as at the beginning. That this is so is indicated also by the fact that even during the normal life of female mice in certain strains, as well as that of animals belonging to other species, a slow but continuous growth of the mammary gland may take place with advancing age. Apparently, rhythmic growth processes may ultimately lead to the development of mammary-gland carcinoma.

Conversely, it may be concluded that the mechanisms which tend to make growth processes strictly cyclic, as well as those mechanisms which underlie the development of refractory states to the action of hormones, are opposed to, and help to prevent the transformation of normal into cancerous tissue. It may also be assumed that without these mechanisms spontaneous cancer could develop more frequently under the influence of hormones as well as of other stimuli. Thus, the study of the origin of cancer is, at the same time, a study of the mechanisms which in the normal organism tend to prevent the development of these abnormal growth processes.

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THE COMPLEMENT-FIXING CAPACITY OF THE RABBIT-PAPILLOMA-VIRUS PROTEIN [1]

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In previous reports the results of quantitative studies on the infectivity associated with the papilloma-virus protein (*1*) have been described. The protein purified in small quantities by ultracentrifugal fractionation of many different batches of cottontail-rabbit warts has thus far exhibited a relatively uniform behavior. Within the limits of recognized experimental variation, infectivity has been quantitatively related to the protein. This has been shown in studies on the relation of protein to (1) the incubation period of the growths induced by it (*2*), and (2) the frequency distribution of positive and negative inoculation sites resulting from the introduction of measured quantities of it (*3*). The data, collected under standardized conditions, add to the increasing evidence indicating the identity of the papilloma virus with the papilloma protein. In so far as this interpretation is admissible, the quantitative findings with the protein are acceptable as representative of the behavior and properties of the virus. From the beginning, it has seemed desirable to accumulate data pertaining to biological properties of the virus or protein other than infectivity. Of these the ability to fix complement demonstrated for the virus (*4*) in crude extracts of cottontail-rabbit warts has been of much interest. The purified protein fixes complement under conditions similar to those under which the virus fixes it. During the past 2 years, quantitative studies of the complement-fixing capacity of the protein have been made on many batches of the material. The results obtained have further emphasized the biological uniformity of the purified product and the intimate relation of protein to the virus. In addition, the findings have been of much practical value in the investigations previously reported and in others to be described later. The present paper is concerned with the results obtained in the studies on complement fixation.

MATERIALS AND METHODS

Guinea-pig complement was employed in all experiments. It consisted of 2 units of complement in a volume of 0.2 cc. titrated a few hours prior to its use. The amboceptor was rabbit-antisheep-cell hemolysin diluted with 0.9-percent NaCl solution to contain 2 units per 0.2 cc. Sheep red-blood cells, washed four times and suspended in 5-percent concentration in 0.9-percent NaCl solution, were sensitized by mixing 0.2 cc. of cell suspension with 0.2 cc. of amboceptor. The complement and cell-amboceptor mixtures were obtained from the Wassermann Laboratory of Duke Hospital through the generosity of Mr. H. W. Craig.

Papilloma protein was purified in the usual way by ultracentrifugal fractionation (*1*) of extracts of papillomas from cottontail rabbits. The warts employed for extraction were growths occurring naturally and found on rabbits trapped in Kansas. When removed in the field, the growths were placed in 8-ounce bottles

[1] *This investigation was carried on at the Department of Surgery, Duke University School of Medicine, and was aided in part by a grant from the National Cancer Institute.*

containing a mixture of equal parts glycerin and 0.9 percent NaCl solution. The bottles were kept in the field until filled with warts, and consequently each contained growths from several rabbits. When the bottles were full, they were shipped to the laboratory and stored at 5°–8° C. Under these circumstances the virus activity of the growths seems to be maintained indefinitely. For extraction, after being freed of connective tissue and hair, warts were ground to a smooth paste with sand and extracted, usually overnight in the refrigerator, in 15 times their weight of 0.9 percent NaCl solution. Sand and gross-tissue particles were then removed by twice-repeated low-speed centrifugation.

The purified virus protein was obtained from these extracts by concentration, and purification by fractionation in an air-driven quantity ultracentrifuge. The crude extract was put into 18-cc. collodion tubes in the ultracentrifuge rotor which was then spun up to 9,000 r. p. m. (about 7,000 times gravity) and quickly stopped. The sediment resulting from this operation was discarded, and the supernatant fluid, after being poured into fresh tubes, was ultracentrifuged in a field of about 60,000 times gravity for 1 hour. The supernatant fluid from this first ultracentrifugation was poured off, and the tubes containing sedimented pellets were inverted for a few minutes to allow excess fluid to drain away. The pellets were resuspended in 0.05 M phosphate buffer solution of pH 6.5 with the aid of fine-tipped pipettes. The resulting suspension was cleared of aggregated insoluble material by spinning in the ultracentrifuge at 9,000 r. p. m., as before. This process of alternate centrifugation and ultracentrifugation was repeated routinely 5 times to provide the purified virus used here. The protein obtained by 5 ultracentrifugal cycles was dissolved in 0.05 M phosphate buffer solution of pH 6.5 and assayed by turbidity measurements (*2*) in the photoelectric colorimeter. Stock solutions were then prepared by dilution with 0.9 percent NaCl solution to contain 70 to 100 gamma protein per cubic centimeter. For the tests, serial twofold dilutions of these solutions were prepared with 0.9 percent NaCl solution.

All of the immune sera except one were from domestic rabbits bearing large, confluent papillomas. The exception was a specimen of serum from a cottontail rabbit which had been hyperimmunized. This serum, W. R. 12–S, was kindly supplied by Dr. J. G. Kidd, of the Rockefeller Institute for Medical Research, New York. Of the domestic rabbit sera, one batch, D. R. V., was a pool from several animals. The complement of these sera was inactivated by heating at 56° C. for 30 minutes in closed tubes. None of the sera showed more than a trace of anticomplementary action in preliminary tests with the undiluted material.

In setting up the experiments, the reaction tubes were placed in metal racks arranged in a shallow pan. Cracked ice was heaped in the pan and water added to a level above that expected for the mixtures in the tubes. The tubes were left thus immersed while the mixtures were being prepared. Appropriate dilutions of immune serum in 0.2-cc. volume were first put into the tubes, and 0.2 cc. of complement was then added. Serum and complement were thoroughly mixed by shaking each tube individually. The dilutions of papilloma protein, 0.2 cc. of each, were added, and the tubes again shaken. All were then placed in the icebox at 5° C. and left for 3 hours. Sensitized sheep cells, 0.2 cc. of 5-percent suspension of cells plus 0.2 cc. of antisheep-cell hemolysin, were put into the tubes, bringing the total volume of the mixtures to 1.0 cc. The tubes were then incubated

in a water bath at 37.5° C. for 1 hour.

Readings of the results were made immediately after incubation. Complement fixation was recorded at ++++, complete; +++, 75 percent; ++, 50 percent; +, 25 percent; and 0, no fixation.

Appropriate controls were included in each experiment.

COMPLEMENT-FIXING CAPACITY OF THE PROTEIN

A total of 25 experiments were made with 8 different sera and 14 individual preparations of protein. In 17 experiments a series of twofold dilutions of protein were set up against successive twofold dilutions of serum, as shown in table 1. The remaining 8 experiments were made with twofold dilutions of protein tested only against those dilutions of serum found to be optimal in previous repeated tests. In every experiment the total quantity of virus required to bind just 2 units of complement when mixed with optimal quantities of antiserum, was determined. This amount was found by interpolation between actual test quantities when necessary. In some experiments, in order to conserve serum, mixtures necessitating lower dilutions of the serum were omitted.

A summary of the results obtained is given in table 2, in which the respective immune sera, protein preparations, and dates of the experiments are indicated. It will be noted that the observations extended over a period of about 17 months. Antiserum, when used in repeated tests over a long period of time was frozen, dried, and stored in a refrigerator. With the exception of material II, each batch of protein was studied within 3 days after purification. Protein II, however, had stood in concentrated state in the refrigerator at 3° to 5° C. for several weeks before its use in experiment 1.

Although the tests were made on the basis of 2 units of complement per reaction tube, the results are considered in terms of complement-fixation units, or the amount of protein which just bound 1 unit of complement in the presence of optimal quantities of antiserum.

Under the standard conditions observed, the amount of protein required to fix 1 unit of complement was found to be 0.95 ± 0.06 gamma, or $10^{-6.023}$ gm., on an average. The coefficient of variation of individual estimates was ± 29.8 percent. This variation in individual experiments was remarkably low considering the fact that biological reagents were employed and that these were made up under routine conditions over a period of 17 months. The values reported here are not considered to be absolute, since variations in technique may alter the value of the complement-fixation unit. However, with the standard technical procedures employed it is evident that fairly consistent results, within known limits of variation, may be obtained.

TABLE 1.—*Fixation of complement by protein XVIII with serum W. R. 12–S*[1]

Total protein (gamma)	Serum dilution							
	0	1:2	1:4	1:8	1:16	1:32	1:64	1:128
0 (control)	0	0	0	0	0			
16.0	++++	++++	++++	++++	++++	+++	0	0
8.0	++++	++++	++++	++++	++++	+++±	++	0
4.0	++++	++++	++++	++++	++++	+	0	0
2.0	+++±	++++	++++	++++	++++	++	++	0
1.0	+++	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0

[1] In experiment 9, table 2.

TABLE 2.—*The quantity of purified papilloma protein required to fix 1 unit of complement*

Experiment No.	Date	Immune serum	Protein preparation	Protein required to fix 1 unit of complement
	1938			*Gamma*
1	Sept. 8	D. R. A	II	0.33
2	Oct. 5	do	V	.90
3	Oct. 6	D. R. 495	V	.54
4	Oct. 7	D. R. B	V	.90
5	Oct. 27	D. R. 495	VIII	1.08
6	Nov. 4	D. R. A	X	.81
7	do	D. R. B	X	.92
8	do	D. R. 495	X	.92
	1939			
9	Jan. 18	W. R. 12-8	XVIII	1.00
10	do	do	XVIII	1.00
11	Jan. 31	D. R. 495	XX	.75
12	Feb. 3	do	XX	1.25
13	Feb. 10	do	XXIII	1.37
14	Feb. 14	do	XXIII	1.25
15	do	do	XXIII	.90
16	Feb. 15	do	XXIII	1.25
17	Mar. 7	D. R. 496	XXIV	1.00
18	Mar. 9	D. R. 848	XXIV	1.25
19	Mar. 28	do	XXVI	.68
20	Mar. 31	do	XXIX	.78
21	Sept. 27	do	XXXI	1.56
22	Nov. 7	do	XXXIV	.62
23	Nov. 9	do	XXXIV	1.25
	1940			
24	Jan. 9	D. R. 2326	XXXIX	.75
25	Feb. 13	D. R. V	XL	.75
	Mean			0.95±0.06

Standard deviation, ±0.283±0.040.
Coefficient of variation, ±29.8±4.2.

It should be noted that the observed variations were not definitely related to the behavior of individual protein preparations or antisera. For instance, the complement-fixing unit of protein V varied from 0.54 to 0.90 gamma. Protein XXX-IV was tested twice with the same antiserum; on one occasion the unit was 0.62 gamma, and on the other 1.25 gamma. Antiserum 495 was used with five different batches of protein, and the variations in the value of the unit were from 0.54 to 1.37 gamma. From inspection it looks as if slight differences might exist. For instance, it seems that the complement-fixing capacity of protein V was greater than that of protein XXIII. On the other hand when the mean values for these two materials, 0.78 and 1.19 gamma, respectively, are compared by methods appropriate for small samples (*5*), the difference between them is not greater than that which could occur because of random sampling ($t=2.637$; $n=5$; $P=0.047$). In general, the observed differences then appear to follow a random distribution, and this is substantiated by the symmetry of the frequency distribution shown in table 3 and figure 1. With this recognized

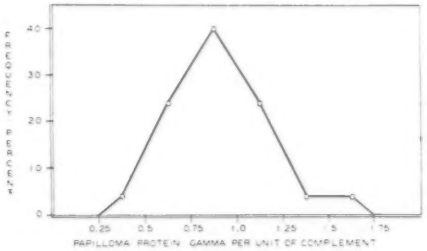

FIGURE 1.—Frequency polygon showing the variation in the quantity of papilloma protein per complement-fixation unit.

degree of variability, therefore, the complement-fixation unit may be defined as 0.95 gamma, or $10^{-6.023}$ gm., of papilloma protein.

TABLE 3.—*Frequency distribution of papilloma-protein quantities fixing 1 unit of complement*

Papilloma protein		Frequency	Percent
Class interval	Mean		
Gamma	*Gamma*		
0.26–0.50	0.375	1	4
.51– .75	.625	6	24
.76–1.00	.875	10	40
1.01–1.25	1.125	6	24
1.26–1.50	1.375	1	4
1.51–1.75	1.625	1	4
Total		25	100

RELATION OF THE COMPLEMENT-FIXING UNIT TO THE INFECTIOUS UNIT

In earlier work a study was made of the frequency distribution of positive and negative inoculations with graded doses of papilloma protein. The amount of pro-

tein required to produce warts at 50 percent of the sites inoculated with it and none at the remaining sites was found to be fairly constant when standardized procedures were employed. On the basis of these observations a unit for the numerical expression of papilloma-protein infectivity was chosen as that amount in the inoculum of standard volume, 0.1 cc., which gave this 50-percent point result. The infectious unit was found to be, on an average, $10^{-8.355}$ gm., or 0.00441 gamma, of protein. Since for practical purposes the statistical averages may be considered as constant, the ratio of complement-fixation unit to infectious unit may be established on the basis of a criterion common to both infectivity and complement-fixation studies, namely, protein quantity. The ratio of complement-fixation unit to infectious unit is, therefore, 0.95 gamma: 0.00441 gamma or approximately 215:1.

It has not been practical to make simultaneous studies of complement-fixing capacity and infectivity on the same protein material in more than a few instances. In two experiments made for the purpose of determining the infectious unit, simultaneous tests were made of the complement-fixing capacity of the protein used in each case. The results with these two studies are summarized in table 4. The ratios in the two experiments were 260:1 and 295:1, respectively. It should be pointed out that the protein values obtained for the complement-fixation units in these individual experiments were among the highest values obtained and that ratios as great as these might be expected to occur less frequently than ratios nearer the mean result of 215:1.

The complement-fixing unit of protein, $10^{-6.023}$ gm., is a quantity which, in a volume of 0.1 cc., would be expected to induce warts appearing after a mean incubation period of 16.9 days. The complement-fixing unit can be correlated on this basis with previously described standard data pertaining to incubation period (*2*) and the frequency distribution (*3*) of positive inoculations of the protein. This correlation can be shown graphically in a straight line drawn horizontally across the nomogram of figure 2 of a recent publication (*3*). The line would pass through the ordinate corresponding to 16.9 days parallel with the base of the nomogram.

TABLE 4.—*Ratio of the complement-fixing unit to the 50-percent point infectious of papilloma protein*

Protein preparation	Protein per complement-fixing unit	Protein per 50-percent point infectious unit	Ratio of complement-fixing unit to 50-percent infectious unit
	Gamma	*Gamma*	
XXIV (a)	1.00		
XXIV (b)	1.25		
Average	1.125	0.00433	260:1
XXXI	1.56	.00528	295:1

DISCUSSION

The results show the existence of a fairly constant relation between protein and the complement-fixing capacity of the different purified preparations. With individual determinations, the results in two-thirds of the instances should fall within approximately $\pm$ 30 percent of the mean found here. The studies were made on 14 different samples of protein over a period of more than a year, and the observed variations are thus related not only to differences in the protein but also to variations inherent in the method and the reacting constituents included in it.

The quantitative correlation of complement-fixing power and protein is of immediate value in its practical application to the study of the purified material. Within the limits of the variations noted, the reaction is suitable for rapid biological

assay of the protein. In many experiments the infectivity of the protein has been found constant within the limits of the methods for infectivity titration. A quantitative relation exists, then, between infectivity and complement-fixing power, and the infectivity can be determined on the basis of the latter property as accurately as with direct infectivity measurements as long as the conditions under which the relation was established, are observed.

The results obtained with the two biological methods thus far applied to assay of the protein, namely, infectivity titration and complement fixation, have yielded no evidence of definite differences in the state or behavior of various samples.

No methods are yet available for examination of the possibility that noninfectious or inactivated papilloma-protein molecules or other inert associated material may have contributed to the fixation of complement. It has been found that papilloma protein inactivated by alkali and by heat retains complement-fixing power. Further, noninfectious papilloma protein produced for instance by the action of alkali, may be soluble and of size indistinguishable by ultracentrifugal analysis from that of the infectious protein. Thus such noninfectious derivatives are subject to concentration and purification by the same procedures used in purifying the infectious protein. It is, therefore, not inconceivable that virus inactivated biologically and present in the cottontail-rabbit warts may have been included and concentrated in the ultracentrifugal procedures providing the purified preparations. However, within the limits of the variations inherent in the methods or in the criteria applied for interpretation, the protein has been uniform in character. The data provided by the complement-fixation test, as those obtained in infectivity titrations, are subject only to very general interpretation with respect to implications concerning the state or purity of the material studied. It is evident that procedures involving the extent of variation seen in such results are of little value in the estimation of chemical purity. From the biological point of view, however, the results of both infectivity measurements and the present ones of complement fixation show remarkably little variation and that which occurs is uniform.

SUMMARY

In 25 experiments, the complement-fixing capacity of 14 different samples of purified papilloma protein was determined. The mean quantity of protein required to fix 1 unit of complement was found to be 0.95 ± 0.06 gamma, or $10^{-6.023}$ gm., and the coefficient of variation of individual estimates was ± 29.8 percent. The ratio of complement-fixing unit to the 50-percent point infectious unit found in previous work to be $10^{-8.355}$ gm. of protein was 215 : 1.

Within the limits of the methods used, the results provided no evidence of significant differences in the behavior of various preparations of protein. From the biological point of view the protein behaves uniformly, and there is evidently a close relation between the infectivity and the complement-fixing power associated with it. Complement-fixation tests may thus be employed, not only in the biological assay of the protein, but also in indirect measurement of infectivity. This is of especial value in preliminary tests for setting up experiments in which specific regions of protein infectivity must be known in advance.

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THE RATE OF TURNOVER OF THE LECITHINS AND CEPHALINS OF CARCINOSARCOMA 256 AS MEASURED BY RADIOACTIVE PHOSPHORUS [1]

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INTRODUCTION

The phospholipid metabolism of neoplastic tissues has been the subject of several investigations. By the use of elaidic acid, Haven (*1*) showed that the phospholipids of carcinosarcoma 256 resembled those of muscle rather than those of liver in their rate of turnover. Jones, Chaikoff, and Lawrence (*2*), employing radioactive phosphorus, found the phospholipid turnover of four tumors (mammary carcinoma, lymphoma, lymphosarcoma, and sarcoma 180) resembled that of liver, kidney, and intestine rather than that of muscle. In subsequent work on carcinosarcoma 256, Haven (*3*) found results opposite to those using elaidic acid, namely, that the phospholipid turnover when radioactive phosphorus was used resembled that of the phospholipids of liver rather than that of muscle. This difference in rates of turnover of phospholipid as measured by the two biological tracers, elaidic acid and radioactive phosphorus, seems significant since the work was done by the same investigator on the same type of tissue and the same methods for isolation and measurement of the phospholipids were used. Sinclair (*4*) has recently pointed out that kidney phospholipid shows a similar difference in rate of turnover as measured by elaidic acid by him (slow turnover), and as measured by radioactive phosphorus by Artom et al. (*5*, *6*, *7*), and Perlman, Ruben, and Chaikoff (*8*) (rapid turnover). This fact constitutes evidence for the idea advanced by several groups (*4*, *9*, *10*) that phospholipids may have at least two functions, one concerned with phosphoric acid and the other with fatty acid metabolism. By inoculating two different types of tumor in the same animal, Jones, Chaikoff, and Lawrence (*11*) showed that each tumor maintains a characteristic phospholipid turnover which seems to be independent of the metabolism of the host.

The above studies of phospholipid turnover have all been carried out on the total phospholipid fraction of each tumor. The rate of turnover of the individual phospholipids, lecithin and cephalin, has been determined in various organs and tissues of the normal rat by Chargaff (*12*, *13*) using radioactive phosphorus, and by Sinclair (*4*, *14*, *15*) using elaidic acid. While both found that lecithin was formed at a somewhat faster rate than cephalin in liver, Sinclair found that lecithin was formed at a faster rate than cephalin in muscle and Chargaff found lecithin and cephalin formed at approximately the same rate in the carcass. Only in brain was cephalin found by Chargaff to be formed at a faster rate than lecithin. No similar studies on the lecithins and cephalins of tumor tissue have been made with either biological tracer. The present investigation was undertaken in order to determine the rate of turnover of the lecithins and cephalins of carcinosarcoma

[1] *From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y. The substance of this paper was presented before the American Society of Biological Chemists at New Orleans, March 1940* (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133**: *xl 1940*). *This investigation was aided by a grant from the Rockefeller Foundation.*

256. In addition, we wished to determine the lecithin:cephalin ratio of this tumor in order to confirm the 40:60 ratio found by MacLachlan (unpublished work from this laboratory) on phospholipids from peripheral tumor tissue.

MATERIALS AND METHODS

TUMOR

The tumor used throughout these experiments was carcinosarcoma 256. Young male albino rats weighing from 70 to 100 gm. were inoculated subcutaneously in the groin by the trochar method. The tumors were from 3 to 8 weeks old at the time the dose of radioactive phosphorus was administered. Only those rats bearing tumors which were growing at a normal rate and which were visibly free from necrotic spots were used.

PREPARATION AND ADMINISTRATION OF DISODIUM HYDROGEN PHOSPHATE SOLUTION CONTAINING RADIOACTIVE PHOSPHORUS

The ferrous phosphide probe[2] which had been bombarded in the cyclotron was dissolved in a small amount of aqua regia, boiled down, and taken up again in nitric acid. The phosphorus was then isolated by the method of Wilson and Kamen (*16*) but without saving the radioactive iron. After the precipitate of magnesium hydroxide was centrifuged off, dilute phosphoric acid was added dropwise to bring the reaction to pH 6–7 (acid to litmus, basic to nitrazine), and the solution made to volume.

Each tumor-bearing rat received by stomach tube 1 cc. of the dose of disodium hydrogen phosphate containing radioactive phosphorus followed by 30 drops of cod-liver oil to hasten absorption. In general, the dose per rat contained less than 5 mg. of phosphorus and possessed an activity of from 30,000 to 65,000 counts per minute on our scale-of-four counter. The 10- and 20-day rats, however, received doses having an activity of from 150,000 to 175,000 counts per minute.

[2] *The author is indebted to Dr. S. N. Van Voorhis of the University of Rochester Physics Department for preparation of the ferrous phosphide probe which was bombarded in the University of Rochester cyclotron, and to Miss Sylvia Levy for preparing the disodium hydrogen phosphate solution from the probe as well as for making the radioactivity measurements on the phospholipid samples.*

ISOLATION OF PHOSPHOLIPID

From 4 hours to 20 days after receiving the dose of disodium hydrogen phosphate containing radioactive phosphorus the rats were decapitated, the tumors removed, and phospholipids were isolated from the whole tumor by the method of Bloor (*17*). The phospholipids were always reprecipitated from petroleum ether in order to free them from adhering neutral fat and cholesterol, and more particularly from inorganic phosphate. The total phospholipid was then redissolved in petroleum ether with the aid of 1–2 drops of 95 percent alcohol, the slight insoluble residue centrifuged off, and the solution made to volume, usually 50 cc. Aliquots were taken, one for determination of the amount of phospholipid by Bloor's oxidative method (*17*), and another for choline determination, while an aliquot containing the bulk of the phospholipid was used for separation of lecithin and cephalin by absolute alcohol.

CHOLINE DETERMINATIONS

The aliquot of the petroleum ether solution set aside for choline determination was placed in a graduated 15-cc. centrifuge tube and the solvent removed by setting the tube containing a boiling tube in a beaker of hot water. The last traces of solvent were removed by lowering the tube into the steam bath and blowing out with a gentle current of air. One cubic centimeter of water was added and the tube heated and stirred in a beaker of boiling water until the phospholipid was

thoroughly emulsified. Three cubic centimeters of saturated barium hydroxide solution was added, the tube containing the boiling tube was closed with a one-hole rubber stopper, and the whole heated for 1 hour in a 15-cc. centrifuge carrier lowered into the steam bath. The solution was then acidified by addition of 0.6 cc. of a 3N solution of hydrochloric acid. After cooling, the coagulated fatty acids were filtered off, washed, and the water solution containing choline made to a volume of 8 cc. Two cubic centimeters of a saturated solution of ammonium reineckate (freshly prepared) was added to the tube, and the choline determination made according to the method of Beattie (*18*). The pink solution of choline reineckate in acetone was read in a Klett-Summerson photoelectric colorimeter. A calibration curve had been made for the colorimeter using known solutions of choline reineckate. The value obtained for choline, multiplied by the factor 6.493, gave the lecithin equivalent to the choline.

SEPARATION OF LECITHIN AND CEPHALIN

The petroleum ether solution containing the main bulk of the phospholipid to be separated was evaporated to a very small volume in a 15-cc. centrifuge tube, absolute alcohol added, the tube chilled, centrifuged cold, and the absolute alcohol was drawn off and combined with the mother liquor. After this process was repeated twice, the separate cephalin precipitates were dissolved in chloroform and combined, and the separate alcohol solutions containing lecithin were also combined. These solutions were evaporated to a volume of 2 cc., and activity measurements were made by the method of Bale, Haven, and LeFevre (*19*). The lecithin and cephalin solutions after counting were made to volume, aliquots taken for determination of amount of phospholipid by Bloor's method (*17*), and an aliquot of the lecithin solution was taken for choline determination.

RESULTS

Choline determinations on 26 samples of tumor phospholipid before separation of lecithin and cephalin gave an average percent of lecithin of 45.8 ± 3.4; cephalin, by difference, would make up about 54 percent of the total phospholipid. Choline values obtained on the lecithin fraction served as a measure of the extent of separation of the lecithin and cephalin. The average value for the percent of total lecithin obtained in the lecithin fraction was 69.9 ± 7.5, a figure which agrees well with the 70–84 percent found by Sinclair (*15*) who also used the absolute alcohol method of separating lecithin and cephalin. The average recovery of the lecithin and cephalin fractions as based on the amount of total mixed phospholipid used in the separation was 100.3 ± 4.4 percent. Table 1 gives the activity of the tumor lecithin and cephalin fractions expressed as percentage of the dose per gram of phospholipid at intervals of from 4 hours to 20 days after administration of the dose.

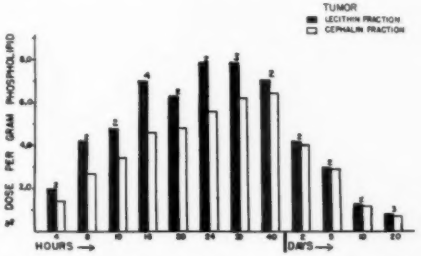

FIGURE 1.—The average percentage of the dose per gram of phospholipid in the lecithin fraction and in the cephalin fraction of carcinosarcoma 256 at various times after administration of disodium hydrogen phosphate containing radioactive phosphorus to tumor-bearing rats. The figures over the bars show the number of animals in each group.

Figure 1 shows the average percentage of the dose at each time. It is apparent from figure 1 that the activity of the lecithin fraction expressed as percent of the dose per gram of phospholipid was greater even at 4 hours than that of the cephalin fraction. While the activity of each fraction increased with time, that of the lecithin fraction did so at a faster rate than that of the cephalin fraction until the peak in activity was reached at 24–30 hours. After this the activity of the lecithin fraction decreased while that of the cephalin fraction continued to increase, reaching its peak at 40 hours. The activity of each fraction then decreased at approximately the same rate.

TABLE 1.—*The percentage of the dose per gram of phospholipid*

Time	Lecithin fraction	Average	Cephalin fraction	Average
Hours:				
4	[1] 2.0		[1] 1.4	
8	4.1	4.2	2.8	2.7
	4.2		2.5	
10	3.5	4.8	2.7	3.4
	6.0		4.1	
16	4.7	7.0	4.0	4.6
	10.5		5.4	
	5.7		3.5	
	7.0		5.3	
20	6.3	6.3	4.3	4.8
	6.3		5.3	
24	8.7	7.8	6.8	5.7
	6.8		4.5	
30	8.8	7.8	6.1	6.2
	7.0		5.7	
	7.6		6.8	
40	7.9	7.0	7.2	6.4
	6.0		5.6	
Days:				
2	4.6	4.2	4.5	4.1
	3.7		3.6	
5	3.5	3.0	3.4	2.9
	2.4		2.3	
10	1.4	1.3	1.3	1.2
	1.2		1.0	
20	.71	.85	.77	68
	.85		.71	
	1.0		.56	

[1] Pooled tumor phospholipids from 2 rats.

DISCUSSION

The 45:55 ratio of lecithin to cephalin may be considered as confirmation of the 40:60 ratio found previously by MacLachlan for this tumor using the modified method of Lintzel and Monasterio (*20*). The slight variation may be regarded as owing to a difference in methods and to the fact that MacLachlan worked with peripheral instead of whole tumor tissue. The significance of this ratio as compared with the 60:40 ratio for liver and the 55:45 ratio for skeletal muscle found by MacLachlan is not yet known.

The incomplete separation of lecithin and cephalin as indicated by the average value of 70 percent for total lecithin obtained in the lecithin fraction, while owing in part to the intersolubilities of these two phospholipids, may also be due to the presence of sphingomyelin in the tumor phospholipid. Sphingomyelin has never been reported present in tumor tissue and if present, would be at least partly removed by dissolving the total mixed phospholipid in petroleum ether in which it is insoluble. However, if it is present and only partially removed by this procedure, its contained choline would be calculated as lecithin in a choline determination on the total mixed phospholipid while sphingomyelin itself would precipitate with cephalin in the absolute alcohol separation. In spite of the fact that a quantitative separation of lecithin and cephalin was not obtained, we feel that the separation was sufficiently good to lend significance to the activity measurements on each fraction.

Figure 1 shows that the rate of turnover of the lecithin fraction of carcinosarcoma 256 as measured by radioactive phosphorus is more rapid than that of the cephalin fraction. As pointed out by Sinclair (*15*), it is evident that the rate of turnover of each phospholipid fraction is too rapid to permit one to ascribe to either phospholipid a purely metabolic or a purely structural function. However, the more rapid rate of turnover of the lecithin fraction would seem to indicate that its function is predominantly metabolic while that of cephalin is largely structural.

Recently, Chargaff (*21*) has shown that aminoethylphosphoric acid is probably not a precursor of cephalin in the body, thus lending additional support to the view previously expressed by him (*12, 13*) that cephalin may be formed from lecithin. The more rapid formation of lecithin than cephalin, as shown in figure 1, may indicate the origin of cephalin from lecithin in this tumor.

SUMMARY

By means of radioactive phosphorus it has been shown that the rate of turnover of the lecithins of rat carcinosarcoma 256 is more rapid than that of the cephalins.

The ratio of lecithin to cephalin in this tumor is approximately 45:55.

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BIOLOGIC TESTING OF CARCINOGENS. I. SUBCUTANEOUS-INJECTION TECHNIQUE

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The tests for carcinogenicity of a given compound are based upon the ability of the compound to produce a malignant neoplasm in an experimental animal. Although recent work on the early effects of the compounds on the mouse skin (*1*), and on other biologic phenomena of the carcinogenic polynuclear hydrocarbons, such as their photodynamic effect upon paramecia (*2, 3*) and upon bacteria (*4, 5*), suggests that simplified and more rapid techniques for evaluating the carcinogenic potency of this group of chemicals may be feasible, evaluations of new compounds based on these tests would have to be substantiated by the criterion of production of tumors in animals.

The term "carcinogenic," although derived from a specific type of malignant growth, by common usage has been extended to include the ability of a substance to induce any type of neoplasm. "Cancerigenic" is preferred by some workers, while "blastogenic" (*6*), "oncogenic" and "tumorigenic" (*7*) (to include benign tumors) have been used by others.

The carcinogenicity of any compound is modified by so many factors that it is necessarily a relative term, applicable only to the specific conditions of the reported experiment.

It is known that some compounds which are carcinogenic to the mouse do not produce tumors in the rabbit, or at least not with comparable doses (*8, 9*). Conversely, data are available which show there are chemicals, such as zinc chloride (*10, 11*) which do not evoke tumors in mice and which are potent, under certain conditions, for the testes of fowl. The carcinogenicity of a compound, therefore, is limited to the species of animal employed in the investigation.

It has been reported that 10-methyl-1,2-benzanthracene (*12*) and 1′,2′-dihydro-4′-methyl-3,4-benzpyrene (*13*) are weakly carcinogenic when applied to the skin of mice, but produce sarcomas readily when injected subcutaneously; 2-methyl-3,4-benzphenanthrene has been determined to be as carcinogenic as 1,2,5,6-dibenzanthracene by the skin-painting technique (*14*) but is only weakly carcinogenic when administered subcutaneously.[1] These differences suggest a tissue specificity, or at least a tissue selectivity, for some of the carcinogens. The term "carcinogenic," therefore, becomes even more limited to the specific tissue of the specific animal species.

The evaluation of the compounds is furthermore modified by the dose and the physical state of the chemical. Since there is an extremely wide range of potency for the various compounds, conclusions are applicable only for the dose employed. Thus, although many chemicals are reported as not being carcinogenic after the subcutaneous introduction of 10 mg. (*12, 13, 15, 16*), there is no assurance that some of these compounds would not have produced tumors at a higher dose, or if they had been injected in solution rather than in crystalline form.

The length of the experiment and the number of animals also determine the

[1] *Andervont, H. B.: Unpublished data. Shear, M. J.: Unpublished data.*

results. Many of the reported evaluations of the compounds are terminated at a year or less, or only a few animals survive the whole period of observation. It is more than likely that some conclusions will have to be revised if the duration of the experiments is prolonged, a larger number of animals is used, and if the compound is administered by several routes.

Further complications occur in the designation of a compound as carcinogenic. In the great majority of investigations with carcinogens, the production of a histologically malignant tumor at the site of application is implied. There are compounds, however, which do not induce tumors at the site of injection but in distant locations. For example, the estrogens,[2] injected subcutaneously or painted on the skin, produce carcinoma of the breast in susceptible mice (*17*) and rats (*18*), and the azo dyes upon subcutaneous, percutaneous, or oral administration produce tumors of the liver in rats and mice (*19, 20*). Although the term "carcinogenic" may well apply to these compounds, opinion is divided concerning the inclusion of estrogens in this group (*17*).

It is perhaps desirable to divide carcinogens into two classes—local carcinogens and distant carcinogens—i. e., compounds which cause tumors at the site of administration and those which induce tumors distant to the application site only. No sharp distinction between these agents is possible; thus, subcutaneously or percutaneously administered hydrocarbons which produce local sarcoma or carcinoma also may induce tumors of the liver (*21*), the breast (*22–24*), or the lungs (*25, 26*).

The discovery that certain polycyclic aromatic hydrocarbons possess the specific property of initiating neoplastic growth in animals (*27, 28*) has led to the synthesis of a large number of related compounds. There are two commonly used methods of testing the local carcinogenicity of these chemicals: (1) The painting procedure, in which the chemical, dissolved in benzene at a concentration of 0.3 percent, is applied twice a week to the intrascapular skin area of mice; and (2) the subcutaneous-injection technique. The former method is used extensively by the London workers (*29, 30*), whereas the latter has been employed in the United States Public Health Service (*12, 13, 15, 16, 31, 32*).

The advantages of the subcutaneous-injection technique are the simplicity and the rapidity with which the original procedure can be performed, and the possibility of more exact and uniform determination of the dosage. Since some compounds probably produce different results by the two routes, it is obvious that neither test is a substitute for, but a complement of the other. Moreover, further determinations, such as the induction of pulmonary tumors in mice (*33*), ultimately will be necessay for complete evaluation of the compounds.

The carcinogenic potency of a series of hydrocarbons has been examined in this laboratory (*12, 13, 15, 16*). The method employed was the single subcutaneous injection into mice of strain A of approximately 2 to 10 mg. of the crystalline compound moistened with glycerin. This procedure and the skin-painting test, if carried out for a sufficient length of time in a sufficient number of animals, give the qualitative answer to whether the particular chemical is able to initiate neoplastic growth at the site of administration or not, but give only a rough approximation of its carcinogenic activity as compared with related compounds.

The exploratory nature of these investigations has sifted the chemicals into the

[2] *Tumors at the site of injection of estrogens have been reported, but the solvents used occasionally induce sarcoma* (*17*).

general classifications of inactive, weakly, or powerfully carcinogenic. It is felt that much valuable information concerning the factors involved in carcinogenesis and the relation of molecular structure to the carcinogenic activity of compounds could be derived from quantitative determinations. The reviews on the subject (*29*, *30*, *34*) indicate the uncertainties of comparing and evaluating results from different laboratories, with the variations in the species and strain of the experimental animals, in the environmental and dietetic condition of the animals, in the mode and route of administration of the compounds, in the quality of the chemical and its physical state or vehicle, in the dosage employed, and in the criteria for recording the results. If quantitative biologic analyses of the compounds are to be obtained, standard test procedures are essential.

During the past 6 years data which have been accumulating in this laboratory have contributed to the understanding of the many factors which modify the induction of neoplasms with chemical compounds. Despite the emphasis on the uncertainties inherent in the procedures and the realization that no single test is adequate, the information available at present is sufficient to summarize these influences upon local carcinogenesis, and on this basis to suggest a standard procedure for the subcutaneous testing of the compounds.

The factors which must be considered in the biologic testing of local carcinogens and related compounds by the subcutaneous-injection technique are given in the following outline:

Factors to be considered in the biologic testing of carcinogens and related compounds

I. Experimental animal
  A. Species
  B. Stain
  C. Age
  D. Diet
  E. Condition of animals
    1. Intercurrent infection
    2. Ulceration at injection site
  F. Environmental conditions
II. Carcinogen
  A. Chemical structure
  B. Purity
  C. Physical state
  D. Vehicle (solvent)
  E. Dose and concentration
III. Mode of administration
  A. Route
  B. Number of injections
  C. Site
IV. Interpretation of results
  A. Diagnosis
    1. Gross diagnosis
    2. Histologic examination
    3. Transplantation
  B. Presentation
    1. Number of animals
    2. Average, 50 percent-latent time, or carcinogenic index.

THE EXPERIMENTAL ANIMAL

The mouse is a desirable animal for the determination of the carcinogenicity of compounds because it is susceptible to tumor induction, readily available and easily maintained, small in size, and hardy. The interest of the geneticists in this animal has led to the development of numerous inbred strains, i. e., genetically homogenous animals obtained by brother-to-sister mating for at least 20 generations (*35*). Although mice of heterozygous make-up are applicable for rough evaluations of the compounds, for more exact details inbred mice are essential (*36*).

The inbred strains vary widely in their susceptibility to carcinogens (*31*). Table 1 shows that mice of strains I or Y are about twice as resistant to tumor induction with 20-methylcholanthrene or 1,2,5,6-dibenzanthracene as mice of strain C_3H. Recent data indicate that mice of strain C are almost as susceptible to the two compounds as are the C_3H animals (*37*).

TABLE 1.—*Susceptibility of seven strains of mice to subcutaneous carcinogenesis (hydrocarbons dissolved in lard)*

Strain of mouse	1, 2, 5, 6-dibenzanthracene				20-methychlolanthrene			
	Dose	Mice	Tumors	Average latent time	Dose	Mice	Tumors	Average latent time
	Milligrams	*Number*	*Number*	*Weeks*	*Milligrams*	*Number*	*Number*	*Weeks*
C_3H	0.8	100	89	18.6	0.8	20	19	9.6
C	.8	31	24	23.2	.8	32	27	16.6
L	.8	22	19	25.2	.8	23	17	16.0
C57 black	.8	34	23	25.1	.8	33	28	19.9
A	[1] 1.6	24	23	21.5	2.0	39	37	10.5
I	.8	44	22	30.9	2.0	38	34	16.1
Y	[1] 1.6	25	21	26.1	2.0	31	22	17.7

Data from Andervont (*31*) and Fieser (*34*).

[1] Administered in 2 doses of 0.8 mg. each.

The male mice of strain C_3H have been found to be satisfactory for the subcutaneous-injection technique of testing carcinogens. The females of the strain should not be used because they develop spontaneous mammary carcinoma early in life (*38*, *39*).[3] The males are hardy, live to be at least 18 months of age, do not ulcerate easily at the site of injection, and are very susceptible to local carcinogenesis with 20-methylcholanthrene or 1,2,5,6-dibenzanthracene. They develop spontaneous hepatoma in about 40-percent incidence, and about 8 percent develop spontaneous primary pulmonary tumors at 16 months of age (*40*);[4] but neither type of neoplasm interrupts the use of the animals for subcutaneous testing.

Other strains of mice have certain advantages over the C_3H mouse, as well as some disadvantages. Strain A mice, for example, are very prone to ulceration at the site of injection. Their great susceptibility to spontaneous and induced primary pulmonary tumors makes lung tissue an admirable medium for testing carcinogens (*25*, *26*, *33*). Certainly when these animals are used, the response of the lungs to the agents deserves as much attention as the local effect.

As indicated above, the response of various tissues to carcinogenic action differs in the inbred strains of mice, and there is no correlation between the susceptibility of the various tissues, i. e., mice susceptible to subcutaneous carcinogenesis may be resistant to induced pulmonary tumors (C57 black strain) and vice versa (I strain) (*31*).

The inbred strains of mice are not a constant test medium. The common ancestry of a strain maintained for many years in two separate laboratories does not exclude the possibility that the two lines have not diverged through selective breeding or mutation. Sublines are not uncommon under such conditions (*41*, *42*). Periodic comparison of the strains is essential if confusing differences in results are to be avoided.

For comparable results, the test animals must be as free from variables as possible. These variables can be encountered in the age, sex, diet, or condition of the mice.

No definite difference in the susceptibility to 20-methylcholanthrene or 1,2,5,6-dibenzanthracene has been established between the male and female mice. The suspected sex influence in mice of the C57 black strain (*31*) has not been substantiated with 3,4-benzpyrene (*43*). Castration does not alter significantly the susceptibility of the mice to subcutaneous carcinogenesis with 1,2,5,6-dibenzanthracene (*44*). Since sex differences may be

[3] [4] *These publications are valid only for the colony of C_3H mice maintained in this laboratory.*

encountered with some new compounds, however, it is desirable to employ only one sex, preferably the male, in order to avoid the occurrence of spontaneous mammary tumors.

Age apparently plays a role in the susceptibility of the mice to carcinogenic compounds, although it is not clear whether the results are due primarily to the age difference or to the dose/weight ratio. In mice of strains C_3H, I, and L, injected subcutaneously with 20-methylcholanthrene in lard, tumors develop earlier in the younger animals (table 2) (*32*). These results are at variance with the observations of other workers (*45*, *46*), so they cannot be stated to be definite. Since the age or the weight of the animals may influence carcinogenesis, these factors should be controlled by the use of mice of the same age and weight. It has been customary in this laboratory to inject mice when they are from 2 to 3 months of age.

TABLE 2.—*Effect of age of mice upon subcutaneous carcinogenesis with methylcholanthrene in lard*

Strain	Mice	Age	Weight	Dose	Tumors	Average latent time
	Number	*Months*	*Grams*	*Mg.*	*Number*	*Weeks*
C_3H	19	1.5	17	0.8	19	8.3
Do	11	5	35	.8	11	9.4
Do	21	11	38	.8	20	10.0
Do	16	17	38	.8	14	10.8
I	10	1.5	16	1.0	10	17.4
Do	11	5	25	1.0	6	22.5

Data from Shimkin (*32*).

Foster nursing, which exerts an extraordinary influence upon the appearance of spontaneous mammary tumors in mice (*47*, *48*), does not affect the incidence or the latent period of carcinogenesis in male mice injected subcutaneously with 20-methylcholanthrene (*37*).

The influence of diet upon carcinogenesis has recently attracted attention. The detoxification and elimination of 20-methylcholanthrene and 3,4-benzpyrene by conjugation with sulfur-containing amino acids have been postulated from the effects of supplying or withholding cystine or methionine from the diet of rats fed with the compounds (*49*, *50*). It has been stated (*51*) that in underfed mice fewer tumors, both spontaneous and induced, are formed and at a later time than in full-fed animals. Up to the present time, animals at this laboratory have been maintained on a commercial diet,[5] but a constant diet with known proportions of known, adequate constituents would be more desirable. When this is not feasible, it is suggested that the diet of the animals be specified, and that the animals be maintained on the same diet in all comparative experiments.

The physical condition of the mice, as a manifestation of their diet and environment, necessarily influences the results. The early death of a considerable percentage of animals negates the accuracy of the data. The effect of intercurrent infection on the development of chemically induced tumors in mice which survive such infection has not been elucidated. Ulceration at the site of injection appears to inhibit carcinogenesis (*12*); large ulcers impair the health of the mice, and some of the administered material may escape from the injection site. For this reason, mice of strains C_3H, C57 black, or L, which are more resistant to ulceration, are preferable for subcutaneous injection to mice of strains A, C, or I.

The environment in which the experimental animals are maintained may also influence the time of appearance and the incidence of tumors. Thus, it is suggested [6] that tumors induced with 20-methylcholanthrene appear earlier in C_3H mice kept

[5] *Purina dog-chow pellets.*

[6] *Wallace, E. W., and Mills, A. C.: Private communication.*

in a temperature of over 90° C. than in mice living in a temperature of 60°.

THE CARCINOGEN

The commercially available hydrocarbons and azo dyes usually contain variable amounts of impurities. The 1,2,5,6-dibenzanthracene impurity, which may amount to up to 20 percent of the total weight, is not carcinogenic when applied to the skin of mice (*34*). The impurity isolated from 20-methylcholanthrene is not carcinogenic subcutaneously, nor does it influence the incidence or latent period of carcinogenesis when mixed with the pure hydrocarbon.[7] A variable proportion of inert material in a chemical, however, introduces considerable error in quantitative experiments. It is also possible that impurities of other compounds may exert some action or be carcinogenic. The melting point should be given as an indication of the purity of the compounds. It is also advantageous to mention the method of preparation and of the purification.

The physical state of the injected hydrocarbon has been demonstrated to influence the incidence and the latent period of carcinogenesis. With 1, 2, 5, 6-dibenzanthracene, tumor formation is slower when the chemical is introduced as a horse-serum dispersion, adsorbed on charcoal or as crystals in glycerin, than when it is dissolved in lard (table 3) (*52*, *53*). The dissolved state appears to be the most efficient in eliciting subcutaneous tumors.

The solvent exerts a definite effect upon the latent period and the incidence of tumors induced with the hydrocarbons (table 4). Certain extracts of mouse tissues inhibit the development of tumors with 3,4-benzpyrene (*43*, *54*). Significant

[7] *Lorenz, E., and Shimkin, M. B.: Unpublished data.*

TABLE 3.—*Effect of the physical state of hydrocarbon upon subcutaneous carcinogenesis with 1,2,5,6-dibenzanthracene in C_3H male mice*

Physical state of hydrocarbon	Dose	Mice	Tumors	Average latent time
	Mg.	*Number*	*Number*	*Weeks*
Lard solution	1.0	45	41	18.1
Horse-serum dispersion	1.0	50	44	24.6
Adsorbed on charcoal	2.0	11	10	22.6
Crystals in glycerin	1.6–2.0	34	24	24.6

Data from Andervont and Lorenz (*52*, *53*).

differences are obtained in the appearance of sarcoma when different lots of lard are used as solvents for 20-methylcholanthrene (*55*) or 3,4-benzpyrene.[8] Because of this variability, and because sarcomas have been reported to develop at the site of injection of lard in rats (*56*), and of sesame oil in mice (*17*), lard and similar animal or plant materials of variable composition are not adequate solvents for pure hydrocarbons. In an attempt to offer a better solvent of known chemical composition, it was found that the glyceride, tricaprylin, is an excellent compound for this purpose (*55*). As with the compound tested itself, the relevant data on the solvent, such as its melting or freezing point, source, and method of preparation should be mentioned.

TABLE 4.—*Effect of solvents upon subcutaneous carcinogenesis with 0.5 mg. 20-methylcholanthrene in C_3H male mice*

Solvent	Mice	Tumors	Average latent time
	Number	*Number*	*Weeks*
Tricaprylin	39	39	9.0
Tricaprylin-trilaurin	20	20	9.7
Butyl stearate	25	24	10.7
Lard, sample B	76	74	10.8
Tricaproin	20	19	12.4
Lard, sample A	38	35	15.5
Butyl phthalate	16	11	21.3

Data from Shimkin and Andervont (*55*).

Although there are certain advantages in injecting the crystalline material (*12*,

[8] *Shear, M. J., and Leiter, J.: Unpublished data.*

13, 15, 16), the more accurate determinations of the dose and the more rapid appearance of tumors make the dissolved state more desirable for the subcutaneous testing of carcinogens. Most solvents, however, have a limited solubility for the hydrocarbons. In tricaprylin, 5 to 10 mg. per cubic centimeter of the solvent at 40° C. is the limit of solubility for 20-methylcholanthrene or 1,2,5,6-dibenzanthracene (*55*). Since mice do not tolerate subcutaneous injections of much over 0.5 cc. of oil, about 5 mg. of the compounds only can be given at a single time. With weak carcinogens, it is well to repeat the injection, or to have a concurrent group of animals injected with the highest nontoxic dose of the compound in the crystalline form. As high doses of carcinogens and related compounds exert a damaging effect, either general or local, which interferes with tumor formation, lower doses may actually be more efficacious.

Tumor induction is dependent upon the total dose of the carcinogen injected and at high doses is not influenced by the concentration of the compound in the solvent. There is no difference in the incidence or average latent time of tumors whether 1 or 2 mg. of 20-methylcholanthrene is dissolved in 0.1, 0.25, 0.5 or 1.0 cc. of lard.[9]

The administration of only one dose of the compound is not adequate for the determination of its potency. A wide range of doses should be employed, e. g., 0.01 to 10 mg. Preliminary dose-response curves, which are the most reliable means of comparing biologically active materials, can be determined from such data.

It is customary to use the absolute weight of the compound in calculating the dose and the concentration of the administered material. It may be well to use the molecular weight rather than the absolute weight for comparative measurements, especially if the compared chemicals differ considerably in their molecular weights.

Since the concept of purely local action of the carcinogens cannot be maintained in view of the observed general effects (*57, 58, 59*), the adjustment of the dose to the weight of the animals also deserves attention. Mice of the same strain, sex, and age, and kept on the same diet do not vary sufficiently in weight, however, to introduce any considerable error due to this factor.

MODE OF ADMINISTRATION

The procedure usually employed in subcutaneous testing of the compounds is the single subcutaneous injection of the solution into an axillary area. The syringe (1 cc.) and the needle (22 to 25 gage, about 4 cm. long) are sterilized, and the skin of the mouse is daubed with alcohol before the administration. The subcutaneous tissues are entered from the rear back, so that the injection tract is long and slopes downward. The puncture wound is pinched for several seconds after the withdrawal of the needle to prevent the escape of the material. More careful preparation of the puncture site, by shaving the skin and painting it with iodine, and the sealing of the puncture with collodion have not been found necessary.

No definite differences have been encountered (*45*) in the administration of the compound in other subcutaneous sites, and the right axilla is therefore suggested in order to make the locus uniform.

Most of the investigations on the effect of dosage upon carcinogenesis have utilized the multiple-injection method, and it may be necessary for toxic substances or for compounds which are readily eliminated from the body, such as the estrogens. A review of the subject (*31*), however, indi-

[9] *Andervont, H. B., and Shear, M. J.: Unpublished data.*

cates no difference in the results obtained with multiple as compared with single subcutaneous injections of carcinogenic hydrocarbons. An experiment performed with 1, 2, 5, 6-dibenzanthracene to check this conclusion is presented in table 5, and the same results are apparent in the testing of carcinogenic extracts of soot (*60*). The minimal latent periods, the spread of the induction times, and the average latent times, as well as the incidence of tumors are remarkably close in the two groups. Since single injections are as efficient as multiple ones, the former technique is preferable from the standpoint of ease and simplicity (*36*). Moreover, reports (*61*) are accumulating that the long-continued repeated injection of various substances which are not usually considered carcinogenic, such as hydrochloric acid buffered with potassium phthalate, 25-percent solution of glucose, and liver extract, produce subcutaneous sarcomas in mice.

TABLE 5.—*Effect of repeated versus single injections of 1, 2, 5, 6-dibenzanthracene on subcutaneous carcinogenesis in C_3H male mice (hydrocarbon dissolved in tricaprylin)*

Injection	Dose	Mice	Tumors	Average latent time
	Milligrams	*Number*	*Number*	*Weeks*
Single	0.5	20	18	23.4
Weekly	1.5	32	29	24.2

Data from Shimkin and Andervont (*36*).

[1] Administered in 4 weekly doses.

INTERPRETATION OF RESULTS

The diagnosis of subcutaneous tumor is made by weekly palpatory examination of the animals. As soon as a hard mass which cannot be dissipated by pressure is felt at the site of injection, the mouse is marked or placed in a separate cage and permitted to live until it is ascertained that the mass is growing progressively.

With some hydrocarbons, particularly with large doses of 20-methylcholanthrene, a soft doughy mass can be felt over a wide area about the injection site about a week before the mass becomes hard. Rarely, hemorrhagic abscesses, hard encapsulations of the injected material, or hyperplastic lymph nodes are erroneously diagnosed as tumors. The percentage of error of the gross diagnosis can be reduced to almost zero if the animals are permitted to live until the tumor is of fairly good size, for example, 1 by 2 cm. In a series of 260 masses from animals killed as soon as tumor was diagnosed in the gross, there was an error of 7 percent; most of the lesions which were not indubitably malignant tumors on histologic examination showed inflammatory and hyperplastic tissue resembling that which antecedes sarcoma. When similar masses were removed from animals a week later, all of the 76 masses were histologically malignant (*36*).

Two important sources of error must be considered in the evaluation of weak carcinogens, or of the minimal doses of potent carcinogens, with which only a few tumors may be obtained in a large series of animals: (1) The role of the vehicle, or of possible impurities in either the vehicle or the chemical cannot be ignored; and (2) the tumors may be of spontaneous origin. Any subcutaneous adenocarcinoma in a female mouse must be considered to be a spontaneous mammary carcinoma unless the incidence of such tumors is unquestionably higher than in the control group (*22, 24*). Spontaneous subcutaneous sarcomas, in both male and female mice, also have been encountered (*62, 63*).

The great majority of tumors induced subcutaneously by the carcinogenic hydrocarbons are spindle-cell sarcomas, which often contain muscle-cell elements. Squamous-cell carcinomas, probably arising from the contact of the carcinogen

with such epithelial tissues as hair follicles, are also seen. Of 317 tumors induced with 20-methylcholanthrene, 3,4-benzpyrene, or 1, 2, 5, 6-dibenzanthracene, 309 were spindle-cell sarcomas and 8 were squamous carcinomas (*36*). In a similar study (*22*) of 68 male mice in which subcutaneous tumors were induced with the 3 hydrocarbons, 65 were sarcomas, and 3 were squamous carcinomas, without or mixed with sarcoma.

Histologic examination of the masses diagnosed in the gross is, of course, essential for the verification of the results. Moreover, it is always possible that some new carcinogen may evoke a particular type of neoplasm. Further confirmation of the data is possible by the transplantation of the induced tumors into mice of the same strain as those in which the original growth was produced. The use of inbred strains makes it possible to transplant such tissue with uniform success (*64, 65*). This also allows the propagation of tumors of special interest.

The appearance of tumors in the experimental group is recorded weekly, according to the time a hard mass is originally palpable. Such results check closely with the histologic diagnosis and with the transplantability of the tissue (*66*). It had been reported recently (*67*) that there is a high degree of correlation between the growth rate and the mitotic index of the induced tumors and the shortness of the latent period as measured by the appearance of palpable tumors, so that the growth rate of a tumor in the microscopic stage may be the main factor in determining the latent period of carcinogenesis. Whether dependent upon the appearance of the malignant transformation or upon the microscopic growth rate, the evaluation of chemical carcinogens by the criteria of incidence and latent period of the palpable tumors seems to be sound.

The method of presenting the experimental data used in this laboratory is given in table 6. The tumors are enumerated by weeks, or by longer periods when the latent periods are longer. Mice dying of extraneous causes before tumors begin to appear in the particular series are subtracted from the total of the animals injected; thus only the "effective total" (*34*) is given. Any considerable intermittent mortality, no matter at what time during the experiment, negates the quantitative nature of the results.

The most common summary is given in

TABLE 6.—*Induction of subcutaneous tumors in C_3H male mice with a single injection of carcinogenic hydrocarbons dissolved in 0.2 cc. of tricaprylin*

Hydrocarbon	Dose	Mice	Number of tumors per week and time (in weeks)																									Total tumors	Average latent time	50-percent latent time	Carcinogenic index
			6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32+				
Methylcholanthrene	*Mg.*	*No.*																												*No.*	*Wks.*	*Wks.*	
	0.25	20			3	5	4	5	1	1	1																			20	10.1	8.9	140
	.5	20	1	3	3	1	5	5	1	1																				20	9.5	8.0	150
	1.0	20	1	3	3	5	3	3	2																					20	9.1	7.8	155
	2.0	20		1	4	3	2	6	3		1																			20	10.1	8.6	140
Benzpyrene	.25	20						2	1	1	4	2	2	2	2	1					1									18	15.4	13.3	90
	.5	20					1	5	1	3	2	2	1	1	2	1														19	13.8	11.6	100
	1.0	20					1	7	3	5	2		1	1																20	12.5	11.1	115
	2.0	20						9	2	3	2	3					1													20	12.1	11.1	120
Dibenzanthracene	.25	19												2			3	1	2	1	1	2		1	1		1	2		17	24.6	20.6	50
	.5	20									1		2	1			2	1	1	1	3	2	1		2		1			18	23.4	20.3	55
	1.0	20								1	1	1		2		2	1	2	2	1	2	1	2		1					19	21.7	18.3	60
	2.0	18								2	2	3	1		2	2	2	1			1			1						17	17.7	14.8	75
	3.0	16							1	1	3	2	1	2	1		2	2												15	15.9	14.1	85

Data from Shimkin and Andervont (*36*).

the form of average latent time, computed by multiplying the number of tumors appearing each week by the time in weeks after injection, and dividing the sum by the total number of tumors. To avoid the inordinate weight of the few late-appearing tumors, it is perhaps better to use the 50-percent latent time, which is calculated by the same method but only the first half of the group which develops tumors is considered. These latent-period averages do not take into consideration the incidence of tumors, which must be presented separately. For this reason, use can be made of the carcinogenic index (*14*), in which the percentage of mice developing tumors is divided by the average latent period of the whole group in days, and multiplied by a hundred. None of these derivations, however, replaces, or is adequate in the absence of, the complete, detailed results, which are most clearly and concisely presented in tabular form.

Two examples of the application of the technique outlined herein are appended.

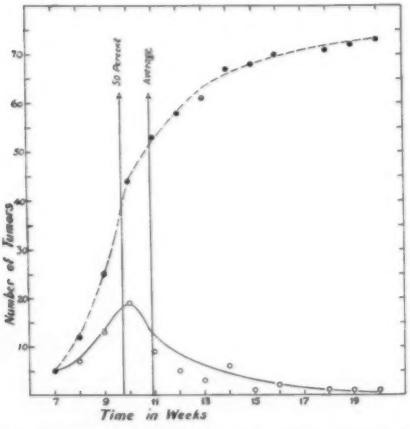

FIGURE 1.—Induction of subcutaneous tumors in 76 strain C_3H male mice with single subcutaneous injection of 0.5 mg. of 20-methylcholanthrene in one sample of lard (*55*).
—○—○— tumors appearing each week;
—●—●— accumulated tumors per week.

The complete details of the experiments have been presented elsewhere (*36*, *55*).

Figure 1 represents the results obtained in a group of 76 C_3H male mice, of the same age and maintained on the same environmental conditions, injected with 0.5 mg. of 20-methylcholanthrene in 0.25 cc. of one sample of lard. The regularity of the data make them susceptible to statistical analysis.

Another experiment, on the comparative carcinogenicity of 20-methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene, is summarized in table 6. The hydrocarbons were dissolved in 0.2 cc. of tricaprylin. The data permit the following conclusions: (1) There is a minimal latent period, as determined by palpation, for each of the three hydrocarbons; (2) the spread of the latent period is shortest for methylcholanthrene and lengthens out with benzpyrene and dibenzanthracene; (3) the average latent times are in the same order; and (4) the maximum effective dose is smallest for methylcholanthrene and greatest for dibenzanthracene. It follows that methylcholanthrene is the most carcinogenic of the three compounds tested, with benzpyrene and dibenzanthracene next in that order.

The experiment raises a most important question—the number of animals adequate for this type of investigation. With 20-methylcolanthrene and 3,4-benzpyrene at the dosage and the physical state employed, 20 mice appear to be sufficient, but the wide spread of the latent period with 1,2,5,6-dibenzanthracene indicates that for statistically significant results a greater number of animals is required. It has been shown (*37*) that the spread of the latent period of carcinogenesis in litter mates of inbred strains is no less than the spread of the latent period in unselected members of the same strain. The results in table 6 were obtained in very susceptible

mice, with relatively high doses of potent carcinogens in a favorable solvent. The spread of the latent period is accentuated when smaller doses of the carcinogen, or carcinogens of weaker potency are injected into less susceptible animals. It is important to note that when the dose of 1,2,5,6-dibenzanthracene was reduced to 0.0025 mg., 2 out of 167 mice developed tumors (*68*). This is a commentary upon the validity of the reports on the lack of carcinogenic effect of compounds which may be comparable to this dose of dibenzanthracene, based upon the study of a dozen animals of which only half survive for a year.

The relative nature of the whole concept of carcinogenicity and the difficulties of the problem are impressive. There are obvious limitations in experiments performed upon only one species, strain, and sex of animal, into which the chemical is injected by one route. There is no single test method which can truly evaluate a process as complicated as that of carcinogenesis. With compounds of special interest, the use of several species, several routes of injection, and various other permutations of technique are indicated. The present suggestions, however, are limited to the preliminary testing of new chemicals by one procedure which is believed will produce accurate and comparable information.

SUMMARY

The carcinogenicity of any given compound is modified by so many factors that it is necessarily a relative term, applicable only to the specific conditions of the reported experiment.

As one standard test, the subcutaneous-injection technique into mice is recommended. Several widely divergent doses of the chemical, from 0.01 to 10 mg., are dissolved in a solvent of known chemical composition, such as tricaprylin. A single subcutaneous injection into the right axilla is made into C_3H male mice, at least 20 per group. The animals are 2 to 3 months of age and are maintained under constant conditions of diet and environment. The presence of tumor is determined by weekly palpatory examination. As soon as a growing hard mass is detected and has reached appreciable size, it is removed for histologic examination. Detailed data for the incidence and the latent periods of the neoplasms are presented.

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BIOLOGIC TESTING OF CARCINOGENS. II. PULMONARY-TUMOR-INDUCTION TECHNIQUE

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Previous communications from this laboratory (*1–13*) reported that primary pulmonary tumors in mice of strain A are induced by 1,2,5,6-dibenzanthracene, 20-methylcholanthrene, 3,4-benzpyrene, 3,4,5,6-dibenzcarbazole, and 2-amino-5-azotoluene (*9*), and that since under certain conditions pulmonary tumors appear in greater frequency and earlier than subcutaneous sarcoma or liver tumors, the lungs of these mice may be a more delicate test object for these carcinogens than the subcutaneous tissues. Quantitative studies (*12*) established that particularly by the intravenous route of administration, the average number of pulmonary tumors induced with 20-methylcholanthrene is directly proportional to the dose of the hydrocarbon and that the number of individual pulmonary tumors increases with progress of time after the injection. It was found (*12*) that there is no exact correlation between the ability of a compound to induce pulmonary tumors and its carcinogenicity for the subcutaneous tissue.

The present paper deals with the response of the lungs of strain A mice to 12 compounds, 9 of which have been described as being carcinogenic subcutaneously or percutaneously, and indicates the applicability of the reaction to the biologic evaluation of the carcinogenicity of these and other chemicals.

EXPERIMENTAL PROCEDURE

The mice of strain A used in these experiments were obtained from the Roscoe B. Jackson Memorial Laboratory of Bar Harbor, Maine. The animals were kept under the same environmental conditions and received a diet of Purina dog-chow pellets and an unlimited supply of water. In most instances an equal number of males and females, kept in separate cages, was used in each group. All mice were injected when they were from 2 to 3 months of age.

The incidence of spontaneous pulmonary tumors in this strain was recorded elsewhere (*12*). Pulmonary tumors are found in animals as young as 3 months, and the incidence rises to 90 percent by the time the animals are 19 months of age (*14*). In mice up to 9 months old the tumors are almost invariably single, whereas in older mice multiple tumors (usually two per pair of lungs) begin to appear; in animals 15 months of age, about 20 percent of the tumors are multiple. The incidence of pulmonary tumors among the control groups maintained concurrently with the experimental series in these investigations, agreed closely with the incidence determined previously.

The animals were killed by cervical dislocation at various periods after the injection of the compounds, and their lungs examined for the presence and the number of pulmonary nodules after fixation in Tellyesniczky's fluid. The subpleural location and the characteristic appearance of the great majority of the nodules (pl. 1) made it possible to count them with gratifying accuracy (*11, 12*).

In most experiments the animals were killed when they were 9 months of age or less, since above that age the incidence of

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PLATE 1.

Pulmonary tumors in strain A mice after an intravenous injection of 0.25 mg. of an aqueous dispersion of 1,2,5,6-dibenzanthracene. *A*, 8 weeks after injection; *B*, 14 weeks after injection; *C*, 20 weeks after injection. × 4.25.

spontaneous pulmonary tumors (about 35 percent) may confuse the results. Moreover, when many individual pulmonary tumors are induced, they tend to coalesce owing to progressive growth and accurate counts become difficult 6 months after injection (fig. 1).

The reaction of the lungs to the carcinogens is surprisingly uniform. With weak carcinogens or with small doses of potent carcinogens, the incidence of pulmonary tumors rises sharply above that of the control group, but few of the lungs contain more than 1 nodule. Thus, 14 weeks after the intravenous injection of 0.25 mg. of 2-methyl-3, 4-benzphenanthrene the lungs contained 0, 0, 1, 1, 2, 0, 1, 1, 0, 2, and 0 nodules; with 0.05 mg. of 20-methylcholanthrene intravenously, the counts were 2, 1, 1, 2, 2, 6, 0, 1, 2, and 2, respectively. When the dose of the carcinogen and the time after injection are increased, or when a more potent carcinogen is used, the multiplicity of the tumors as well as the incidence are increased. Thus, 20 weeks after the intravenous injection of 0.25 mg. of 3, 4, 5, 6-dibenzcarbazole, the lungs were seen to have 8, 3, 5, 13, 8, 3, 5, 3, 3, 4, 7, and 6 nodules per pair; 23 weeks after the subcutaneous administration of 1 mg. of 1, 2, 5, 6- dibenzacridine the lungs had 65, 55, 58, 52, 57, and 95 tumors each. Only the nodules visible macroscopically on the external surfaces of the lungs were counted.

FIGURE 1.—Pulmonary tumors in a strain A mouse, 40 weeks after the subcutaneous injection of 1 mg. of 1, 2, 5, 6-dibenzacridine in 0.3 cc. of sesame oil. The nodules are confluent and difficult to count. × 4.25.

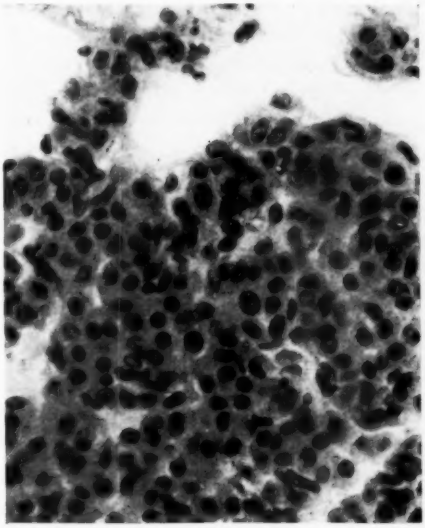

FIGURE 2.—Histologic appearance of a typical pulmonary tumor in a strain A mouse, 20 weeks after an intravenous injection of 0.25 mg. of an aqueous dispersion of 2-methyl-3, 4-benzphenanthrene. Haematoxylin and eosin. ×385.

Lynch (*15*) suggested that the genetic factor which determines the multiplicity of pulmonary tumors in mice may be distinct from the factor which determines the incidence of the tumors. Numerous experiences in the induction of these neoplasms with carcinogens, as well as observations on the spontaneous tumors, however, indicate that the multiplicity of the tumors is the more advanced or stronger response to a longer acting or stronger stimulus.

Sections of the lungs were stained with haematoxylin and eosin for histologic examination. The tumors have been

described in previous publications (*1*, *7*, *11*), and it is only necessary to state that the pulmonary tumors evoked by all the carcinogens referred to in this paper were similar in the site and gross and histologic appearance, and were of the same adenomatous structure as practically all of the spontaneous or induced primary pulmonary tumors seen in strain A mice (fig. 2).

EXPERIMENT 1, INTRAVENOUS INJECTION

In table 1 are listed the hydrocarbons used in experiment 1, together with their melting points, the route of injection, and literature reference.

The carcinogenic potency of the compounds for the subcutaneous tissues are found in the reports of Shear (*16–19*), the London group (*20*), and Boyland and Brues (*21*); and for the skin the data have been summarized by Iball (*22*). The results were obtained on few animals and are not entirely suitable for exact comparisons, as the work was aimed at determining whether the compounds were very active, fairly or weakly active, or negative in eliciting the neoplastic response. It has been established, however, that 3,4-benzpyrene is more potent than 1,2,5,6-dibenzanthracene for the subcutaneous tissue (*23*) and skin (*24*). Generally, it may be said that there is good agreement as to the relative potency of the above compounds tested by the two routes, except for 2-methyl-3,4-benzphenanthrene which is as potent as 1,2,5,6-dibenzanthracene for the skin (*22*) and much weaker when introduced subcutaneously.[1] Thus, 3,4-benzpyrene can be designated as highly active; 3,4,5,6-dibenzcarbazole, 4′-methyl-3, 4-benzpyrene, and 1,2,5,6-dibenzacridine as of medium potency; and 1,2,5,6-dibenzacridine and 15,16-benzdehydrocholanthrene as of low activity for the subcutaneous and/or the cutaneous tissues. The two remaining compounds, 3-methoxy-10-propyl-1,2-benzanthracene and 1,2-benzanthracene are non-carcinogenic.

TABLE 1.—*Nine polycyclic aromatic hydrocarbons and related compounds used in inducing pulmonary tumors in mice when injected intravenously*

Compound	Melting point	Reference, subcutaneous route
	° *C., corr.*	
1,2,5,6-dibenzanthracene	226.0–266.8	(*16*)
3,4-benzpyrene	178.6–179.0	(*16*)
3,4,5,6-dibenzcarbazole [1]	156.5–157.5	(*21*)
1,2,5,6-dibenzacridine [1]	227.0–227.8	(*20*)
2-methyl-3,4-benzphenanthrene [1]	70.5– 71.1	
4′-methyl-3,4-benzpyrene [2]	221.0–221.5	(*18*)
15,16-benzdehydrocholanthrene [2]	181.0–181.3	(*17*)
3-methoxy-10-propyl-1,2-benzanthracene [2]	134.0–135.0	(*19*)
1,2-benzanthracene	160.0–161.0	(*19*)

[1] Procured through the kindness of Prof. J. W. Cook. Melting points were determined by Dr. J. L. Hartwell.
[2] Procured through the kindness of Dr. M. J. Shear.

In previous studies in which carcinogens were injected intravenously (*6*, *11*, *12*), use was made of dispersions of 20-methylcholanthrene and 1,2,5,6-dibenzanthracene in horse serum saturated with cholesterol, prepared by Dr. Egon Lorenz (*25*). Here, Boyland's method (*26*) of preparing aqueous dispersions of hydrocarbons was tried. The chemicals were dissolved in acetone (C. P.) to form a saturated solution which was poured into distilled water and stirred vigorously. A small amount of commercial lecithin or dioctyl ester of sodium sulfosuccinate [2] was added to the mixture as wetting agents. Nitrogen was then bubbled through the mixture until no acetone odor could be detected.

The dispersions were prepared so as to obtain a concentration of 1 mg. of the compound per 1 cc. of water. In many of the suspensions, however, some of the chemical settled out or adhered to the glass, so that the concentration in all instances was not exact and probably was below the 0.1 percent intended.

[1] *See experiment 3.*
[2] *Aerosol O. T., American Cyanamide Co.*

The mice tolerated 0.25 cc. of the majority of the dispersions intravenously with almost no mortality; with 1,2,5,6-dibenzanthracene, 1,2-benzanthracene and 15,16-benzdehydrocholanthrene, however, about 25 percent of the animals died immediately after injection. If the mice withstood the initial procedure, no further mortality was encountered with any of the compounds. The suspensions were stored in the refrigerator at about 0° C. and could be used after agitation many weeks later.

This method of preparing compounds for intravenous injection is not recommended for exact studies because the concentrations are not accurate. It is believed that since all of the dispersions used in this experiment were prepared in the same manner, the results are comparable despite this error.

Equal numbers of male and female mice received a single intravenous injection in a lateral tail vein of 0.25 cc. of the suspensions containing approximately 0.25 mg. of the chemicals. About one-third of each group was killed 8, 14, and 20 weeks later, and the lungs were examined macroscopically for the presence and number of pulmonary nodules.

Results

As summarized in table 2, multiple pulmonary tumors were induced within 8 weeks with 1,2,5,6-dibenzanthracene, 3,4,5, 6-dibenzcarbazole, 3,4-benzpyrene, 15,16-benzdehydrocholanthrene, and 1,2, 5,6-dibenzacridine. The incidence and multiplicity of the tumors rose sharply at 14 weeks after injection, but the relative positions of the compounds remained unaltered. Between 14 and 20 weeks, however, there was only a slight increase in the number of nodules. It appears that by 14 weeks the maximum response was approached with the particular dose of the compounds (about 0.25 mg.).

With 2-methyl-3,4-benzphenanthrene and 4′-methyl-3,4-benzpyrene, the incidence of pulmonary tumors was increased to twice or more than twice that of the untreated controls, and a few of the lungs contained two tumors each. Pulmonary tumors were probably induced with these two compounds, but for indubitable conclusions a larger dose should be used.

Mice injected with 0.25 mg. of 3-methoxy-10-propyl-1,2-benzanthracene or 1,2-benzanthracene had no more tumors of the lung than the untreated controls killed

TABLE 2.—*Response of lungs of strain A mice to a single intravenous injection of 0.25 mg. of aqueous dispersions of carcinogenic hydrocarbons and related compounds*

Compound	Time after injection (in weeks)								
	8			14			20		
	Mice injected	Mice with lung tumors	Average of lung tumors	Mice injected	Mice with lung tumors	Average of lung tumors	Mice injected	Mice with lung tumors	Average of lung tumors
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1,2,5,6-dibenzanthracene	12	12	15.1	10	10	28.2	10	10	30.5
3,4,5,6-dibenzcarbazole	10	7	2.6	10	9	5.2	12	12	5.7
3,4-benzpyrene	10	8	2.3	10	8	4.4	10	10	3.7
15,16-benzdehydrocholanthrene	8	3	2.7	10	8	3.0	10	9	3.0
1,2,5,6-dibenzacridine	10	3	1.3	13	9	2.9	12	11	2.2
2-methyl-3,4-benzphenanthrene	10	2	1.5	11	6	1.7	11	6	1.3
4′-methyl-3,4-benzpyrene	8	2	1.0	11	5	1.1	10	5	1.1
3-methoxy-10-propyl-1, 2-benzanthracene	3	0	0	10	2	1.0	10	1	1.0
1, 2-benzanthracene	10	0	0	10	2	1.0	11	2	1.0
Controls	20	1	1.0	20	3	1.0	19	4	1.0

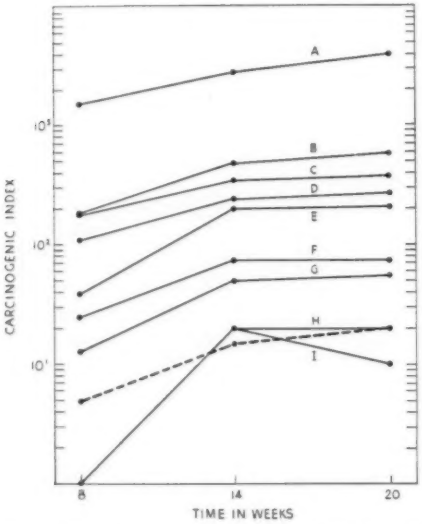

FIGURE 3.—Response of lungs of strain A mice to a single intravenous injection of approximately 0.25 mg. of nine compounds dispersed in 0.25 cc. of water.

Carcinogenic index = percent of mice with pulmonary tumors × average number of pulmonary tumors in positive animals.

Broken line = untreated controls.

A = 1,2,5,6-dibenzanthracene; B = 3,4,5,6-dibenzcarbazole; C = 3,4-benzpyrene; D = 15,16-benzdehydrocholanthrene; E = 1,2,5,6-dibenzacridine; F = 2-methyl-3,4-benzphenanthrene; G = 4′-methyl-3,4-benzpyrene; H = 1,2-benzanthracene; I = 3-methoxy-10-propyl-1,2-benzanthracene.

at the same time, and it is concluded that these two compounds, within the dose and the length of the experiment, did not induce pulmonary tumors in strain A mice.

The data of table 2 are plotted on semilogarithmic paper in the graph (fig. 3). The carcinogenic index (*12*) is derived from multiplying the percentage of mice developing pulmonary tumors by the average number of pulmonary tumors in the positive animals, the times being constant. Figure 3 shows that 1,2,5,6-dibenzanthracene is highly potent in inducing pulmonary neoplasia, being almost as potent as 20-methylcholanthrene, which was assayed previously (*12*). The 3,4,5,6-dibenzcarbazole, 3,4-benzpyrene, 15,16-benzdehydrocholanthrene, and 1,2,5,6-dibenzcarbazole can be designated as of medium potency, whereas 2-methyl-3,4-benzphenanthrene and 4′-methyl-3,4-benzpyrene are of low potency.

It was reported (*12*) that with subcutaneously injected lard solutions of 3,4-benzpyrene and 1,2,5,6-dibenzanthracene there is no parallelism between the local carcinogenic potency and the ability to induce pulmonary tumors, since 3,4-benzpyrene, more potent than 1,2,5,6-dibenzanthracene injected subcutaneously, produced much fewer tumors of the lung. At that time it was concluded that this difference may be in part dependent upon the ability of the agent or its derivatives to be transferred from the locus of injection to the pulmonary tissue.

The results presented here, however, indicate that this difference is due rather to a difference of susceptibility of various tissues to various carcinogens, i. e., tissue selectivity. Subcutaneously, 4′-methyl-3,4-benzpyrene is reported to be about as potent as 1,2,5,6-dibenzanthracene in producing local sarcomas (*18*), yet it is a weak inducer of pulmonary tumors. Conversely, 15,16-benzdehydrocholanthrene was found to be a weak carcinogen subcutaneously (*17*), producing 1 tumor in 14 mice within 11 months after the introduction of 10 mg. of the crystals, yet it is about as potent as 3,4-benzpyrene in producing tumors of the lung when injected intravenously.

The experiment illustrates a proposed method of evaluating the relative carcinogenicity of various compounds. As with any other single technique, it is not a substitute for, but a complement of other tests, and the results obtained with it are not in accord with those obtained by the subcutaneous injection route. The great ad-

vantage of the method is the rapidity (3 months) with which results can be obtained.

EXPERIMENT 2, SUBCUTANEOUS INJECTION, 14 WEEKS

It was noted previously (*12*) that 20-methylcholanthrene is more potent than 1,2,5,6-dibenzanthracene in eliciting pulmonary tumors in strain A mice when injected intravenously; but when the compounds were injected subcutaneously as lard solutions, the latter appeared to be slightly more potent in inducing tumors of the lung.

In order to ascertain whether such differences could be obtained with other compounds and whether the subcutaneous route was applicable in the evaluation of carcinogens by their ability to induce pulmonary tumors, experiment 2 was performed.

Samples of seven of the nine compounds used in experiment 1—3,4-benzpyrene, 1,2,5,6-dibenzanthracene, 1,2,5,6-dibenzacridine, 15,16-benzdehydrocholanthrene, 4′-methyl-3, 4-benzpyrene, 3-methoxy-10-propyl-1, 2-benzanthracene, and 1,2-benzanthracene—as well as 20-methylcholanthrene (m. p. 178°–179.2° C., corr.) were dissolved in tricaprylin, so that 0.1 cc. of the solvent contained 0.5 mg. of the compound. Strain A mice of both sexes were injected in the right axilla with 0.1 cc. of each solution.

RESULTS

Fourteen weeks after the injection, the mice were killed and their lungs examined for the presence and the number of pulmonary nodules. At this time, 9 of 10 mice injected with 20-methylcholanthrene, 3 of 10 mice injected with 3,4-benzpyrene, and 1 of 19 animals which received 4′-methyl-3,4-benzpyrene had sarcomas at the site of injection.

The results, summarized in table 3 show that 1,2,5,6-dibenzanthracene produced the greatest number of pulmonary tumors, with 20-methylcholanthrene next in potency. The reason for the much higher averages, 17.1 and 10.6, than those obtained in previous experiments in which the two compounds were dissolved in 0.25 cc. of filtered lard is obscure, but may be owing to the influence of the solvent.

TABLE 3.—*Response of lungs and subcutaneous tissue of strain A mice 14 weeks after a single subcutaneous injection of 0.5 mg. of various compounds dissolved in 0.1 cc. of tricaprylin*

Compound	Mice	Mice with subcutaneous tumors	Mice with pulmonary tumors	Average of pulmonary tumors
	Number	*Number*	*Number*	*Number*
20-methylcholanthrene	10	9	10	10.6
3,4-benzpyrene	10	3	6	1.1
4′-methyl 3,4-benzpyrene	19	1	5	1.0
1,2,5,6-dibenzanthracene	10	0	10	17.1
1,2,5,6-dibenzacridine	20	0	20	3.0
15,16-benzdehydrocholanthrene	19	0	4	1.2
3-methoxy-10-propyl-1,2-benzanthracene	20	0	2	1.0
1,2-benzanthracene	10	0	0	------

None the less, the relative potency of the two compounds for the lungs when injected subcutaneously is reiterated. The 1,2,5,6-dibenzacridine injected subcutaneously appears to be unquestionably more potent than 3,4-benzpyrene or 15,16-benzdehydrocholanthrene, both of which were slightly more potent than 1,2,5,6-dibenzacridine intravenously, as determined in the single evaluation of experiment 1.

With 15,16-benzdehydrocholanthrene and 4′-methyl-3,4-benzpyrene, the results of the experiment were not conclusive.

The lungs of mice injected with the two compounds reported to be noncarcinogenic did not have any more pulmonary tumors than the untreated animals.

Despite the fact that the subcutaneous dose was at least twice the intravenous one, fewer pulmonary tumors were induced by

the subcutaneous route of administration with all the compounds. With the chemicals tested there is a general agreement as to carcinogenicity for the lungs by the two routes; i. e., compounds which are highly carcinogenic intravenously are also highly carcinogenic for the pulmonary tissue when injected subcutaneously, whereas chemicals which are of medium carcinogenicity intravenously are of medium or low potency when injected by the subcutaneous route. It is apparent, however, that there is sufficient discrepancy to make the results obtained by the two routes of administration not strictly comparable. Whereas aqueous dispersions of the compounds were used for the intravenous injection, oily solutions were used for the subcutaneous injection. The results, therefore, may be owing to the different physical state of the compounds.

The lack of correlation between the local carcinogenicity (production of sarcoma at the site of injection) and the ability to initiate pulmonary tumors is evident.

The subcutaneous route is not so desirable as the intravenous in experiments on the induction of pulmonary tumors because fewer tumors are induced and because local sarcomas may interfere with the determinations. Moreover, if pulmonary tumors are the results of direct contact of the carcinogen with the tissues of the lung, there is greater possibility that the subcutaneous route may be influenced by the transportability and metabolic changes of the chemical, which may or may not be directly involved in the carcinogenic property of the material.

EXPERIMENT 3, SUBCUTANEOUS INJECTION, 40 WEEKS

In experiment 2 the mice were killed 14 weeks after injection, and the results with several compounds were inconclusive probably because of the insufficient length of time. To observe the reaction of the lungs and of the subcutaneous tissues to several compounds over a longer period, experiment 3 was designed.

Three compounds, 1,2,5,6-dibenzacridine, 1,2,7,8-dibenzacridine,[3] and 2-methyl-3,4-benzphenanthrene were dissolved in sesame oil, 1 mg. per 0.3 cc., and injected subcutaneously into the right axilla of strain A mice. The same dose of 3,4,5,6-dibenzcarbazole was toxic, and it was found that 1.0 mg. of 1,2,5,6-dibenzanthracene produced large ulcers in the animals so that the mortality was high and fewer tumors were obtained than had been evoked previously with lower doses. The dose for these two compounds, therefore, was reduced to 0.2 mg. per 0.2 cc. of sesame oil and was injected in the same manner.

The mice were examined weekly for the presence of tumor at the site of injection and were killed when a growing mass was detected. The lungs were examined for the presence of pulmonary tumors. Twenty-three weeks after injection, six mice injected with 1,2,5,6-dibenzacridine, 1,2,-7,8-dibenzacridine, or 2-methyl-3,4-benzphenanthrene were killed in order to observe the reaction in the lungs. The other animals were permitted to live for 40 weeks, when all survivors that had not developed subcutaneous tumors were killed and the lungs examined for the presence and the number of pulmonary nodules.

RESULTS

The results are presented in table 4. When subcutaneously injected, 3,4,5,6-dibenzcarbazole appeared to be more active than 1,2,5,6-dibenzanthracene, in 0.2-mg. doses, in eliciting sarcoma at the

[3] *Referred to as 3,4,5,6-dibenzacridine by the London workers* (20). *Obtained from Prof. J. W. Cook. Melting point, determined by Dr. J. L. Hartwell, was 219.3°–220.1° (corr).*

TABLE 4.—*Response of lungs and subcutaneous tissue of strain A mice to a single subcutaneous injection of five compounds*

[Numerator=mice with pulmonary tumors; denominator=mice killed]

Compound	Dose	Tumors	Time after injection (in weeks)										Summary (40 weeks)					
													Subcutaneous			Pulmonary		
			13	16	19	22	25	28	31	34	37	40	Effective total	Tumors	Average latent time	Mice	Mice with lung tumors	Average of lung tumors
	Mg.													*Number*	*Weeks*	*Number*	*Number*	*Number*
1,2,5,6-dibenzacridine	1.0	Subcutaneous											14	0				
		Pulmonary				6/6						14/14				14	14	70
1,2,7,8-dibenzacridine	1.0	Subcutaneous											13	0				
		Pulmonary				8/6						13/13				13	13	20
2-methyl-3,4-benzphenanthrene	1.0	Subcutaneous				1		1	1		1		18	4	31.0			
		Pulmonary				5/6		0/1	0/1		0/1	8/8				8	8	2.3
3,4,5,6-dibenzcarbazole	.2	Subcutaneous	1	3	5	1	2	2			2	1	20	17	22.0			
		Pulmonary	0/1	2/3	3/5	1/1	2/2	2/2			2/2	3/3				8	8	5
1,2,5,6-dibenzanthracene	.2	Subcutaneous		1	1	2	5	4		1	2		20	16	26.6			
		Pulmonary		1/1	1/1	2/2	5/5	4/5		1/1	2/2	4/4				10	10	35

site of injection, but induced one-seventh as many pulmonary tumors by the fortieth week.

This lack of correlation between subcutaneous carcinogenicity and carcinogenicity for the pulmonary tissue is strikingly demonstrated with the acridines. No subcutaneous tumors were induced in 40 weeks, while the lungs were filled with adenomatous nodules.[4]

Both of the acridines were probably less potent than 1,2,5,6-dibenzanthracene in inducing pulmonary tumors, considering that the dose of the acridines was five times that of the dibenzanthracene. Although the number of pulmonary tumors is directly proportional to the dose of the carcinogen administered (*12*), it should be stressed that widely divergent doses of different compounds cannot be compared in this manner without reservation. It is quite possible that at least by the subcutaneous route there is a threshold dose below which no pulmonary tumors are obtained because no "overflow" of the carcinogen from the site of injection takes place, and a saturation dose, above which no increase in the number of pulmonary tumors can be obtained.

The 2-methyl-3,4-benzphenanthrene, which is reported to be as carcinogenic as 1,2,5,6-dibenzanthracene by the percutaneous method of testing (*22*) was but weakly carcinogenic when administered subcutaneously.[5] The compound is more potent locally than the acridines, yet, as by the intravenous route of administration, it is much weaker in inducing pulmonary tumors.

The experiment shows that the use of the subcutaneous route of injection in the evaluation of carcinogens by the pulmonary-tumor-induction technique requires about 6 months. This is approximately twice the period necessary when the intravenous route is used. Furthermore, as the data of this experiment and experiment 1 indicate, the results obtained by the two routes and

[4] *The London group (20) tested the compounds by the subcutaneous injection, "at fortnightly intervals," of 1 mg. dissolved in 0.3 cc. of sesame oil, and elicited 8 sarcomas in 13 stock mice in 27 to 34 weeks after injection of 1,2,5,6-dibenzacridine; no tumors were found within 44 weeks with the same dose of 1,2,7,8-dibenzacridine.*

[5] *Shear (unpublished results) obtained negative results with this compound, which he injected in crystalline form subcutaneously into strain A mice.*

the two physical states in which the compounds are administered are not comparable.

The value of the pulmonary-tumor test as an adjunct of the subcutaneous-injection technique is also demonstrated; indeed, the susceptibility of the lungs of mice of strain A makes them desirable for the subcutaneous testing of carcinogens despite the disadvantage of their susceptibility to ulceration. By using strain A mice, and by examining their lungs for the presence and the number of pulmonary tumors, two simultaneous determinations are obtained, both of which can be performed on a quantitative basis.

EXPERIMENT 4, 2-AMINO-5-AZOTOLUENE

It was reported previously (*9*) that pulmonary tumors are induced in mice of strains A and C after the subcutaneous injection of 30 to 110 mg. of 2-amino-5-azotoluene. Attention was directed to the fact that a commercial sample with a high percentage of impurities was used. Since then the compound was purified by Dr. J. L. Hartwell, and the experiment repeated with the compound at a melting point of 102.5°–103° C., corrected. In the interim, Morosenskaya (*27*) also has recorded the increase in the incidence of pulmonary tumors in mice of the Russian P–B strain which received 2-amino-5-azotoluene orally, subcutaneously, or percutaneously.

Twenty strain A male mice, 2 months old, received a monthly subcutaneous injection of approximately 10 mg. of 2-amino-5-azotoluene moistened with glycerin. All but one survived the term of the experiment. Twelve mice were killed 8 months after the first injection, when they had received 80 mg. of the compound and were 10 months of age. Seven mice received two more injections of the azo dye (total dose, 100 mg.) and were killed 10 months after the initial injection, or when the animals were 1 year old.

Another group of 21 strain A mice of both sexes received a single intravenous injection of 0.5 mg. of the azo dye dispersed in 0.5 cc. of water according to the technique described in experiment 1. The mice tolerated the procedure without mortality. Half of the animals were killed 14 weeks after the injection, and half were retained until the twentieth week.

RESULTS

Eight months after monthly injections of 10 mg. subcutaneously, 12 mice showed the following neoplastic reaction in the lungs: 1 animal had 6 individual pulmonary tumors; 2 had 3 pulmonary tumors each; 3 had 2 tumors each; 3 had single nodules; and 3 mice were negative for pulmonary tumors. The incidence of 75 percent at the age of 10 months, and the average of 2.3 pulmonary tumors per positive animal are sufficiently above the spontaneous occurrence of 40 percent (of which almost all are single tumors) at that age to conclude that the tumors were induced with the compound.

Of the seven mice which received 100 mg. of 2-amino-5-azotoluene and were killed when they were 1 year old, one had six pulmonary tumors, three had three pulmonary tumors, two had two pulmonary tumors, and one had a single lung nodule.

Since the induction of pulmonary tumors with 2-amino-5-azotoluene has been substantiated with a sample free from impurities, the findings are combined with the results obtained previously and are presented in table 5.

Of the 10 mice which were injected intravenously with 0.5 mg. of 2-amino-5-azotoluene and killed 14 weeks later, only 1 had a single pulmonary tumor. Of the

11 mice killed 20 weeks after injection, 3 had single pulmonary tumors. These incidences were not above those found in the controls (experiment 1), and it is concluded that pulmonary tumors were not induced by the procedure. No comparison is possible, of course, with the mice injected subcutaneously which were treated repeatedly and with a dose 160 times or more greater than the one used intravenously.

TABLE 5.—*Response of lungs of strain A mice to subcutaneous injections of 2-amino-5-azotoluene*

Dose	Time after first injection	Number of mice	Mice with lung tumors	Average number of lung tumors
Mg.	*Months*			
20-40	2-5	5	2	2
50-70	5-8	6	5	1.5
80	8-9	14	10	2.3
90	9-10	5	4	3.5
100	10-11	8	8	2.6
110	11+	7	7	5.7

The induction of pulmonary tumors with 2-amino-5-azotoluene is of special interest. In the previous experiments, the chemicals which were found to be non-carcinogenic to the lungs were reported to be also non-carcinogenic for the subcutaneous tissues or the skin, and the compounds which induced pulmonary tumors were found, or had been reported, to induce tumors at the site of subcutaneous injection or skin painting. Even this qualitative correlation between the response of the lungs and the subcutaneous or skin tissue is not present with the use of 2-amino-5-azotoluene. No subcutaneous sarcomas at the injection site (*28, 29*) or carcinomas after painting the skin (*30*) have been obtained with this compound in mice, although it produces primary liver tumors in the animals.

In this instance, therefore, a compound which does not produce local tumors is found to induce primary pulmonary tumors in strain A mice.

DISCUSSION

The lungs of strain A mice can be used as a medium for testing the carcinogenicity and the relative potency of a wide range of cancer-inducing chemicals. The intravenous route of administration is preferred in most instances because the reaction is greater and more rapid, tumors at the site of injection are precluded, and possible variations due either to destruction of the chemical before it can reach the lungs or to its inability to dissolve and come in contact with the lungs, are avoided.

Aqueous dispersions of the compounds suitable for intravenous injection can be prepared by dissolving the chemical in acetone, adding the solution to water to which a wetting agent is added, and driving off the acetone by heat or by bubbling nitrogen through the mixture. The mice are killed at regular intervals, for instance, 8, 14, and 20 weeks after injection, and the incidence and the number of individual pulmonary tumors per animal are determined macroscopically after the lungs have been fixed in Tellyesniczky's fluid. Quantitative determinations can be obtained by the method.

Perhaps the greatest disadvantage of the biologic evaluation of carcinogenicity by the pulmonary-tumor-induction technique is the lack of a clear end point, since strain A mice develop pulmonary tumors spontaneously at an early age. But since all strains of mice develop the neoplasm if observed for a sufficient length of time, and since the susceptibility of the animals to induced pulmonary tumors is parallel to their susceptibility to spontaneous pulmonary tumors (i. e., mice most susceptible to induced tumors of the lung are also most susceptible to spontaneous pulmonary tumors, and vice versa), this objection cannot be overcome by the use of more resistant mice. Thus, if strain C57 black mice, which are very resistant to pulmon-

ary tumors, are utilized, much larger doses of the carcinogen and a much longer period of time for the induction of pulmonary tumors would be required (*8, 11*). Although strain A mice are most susceptible to pulmonary neoplasia after the injection of 1, 2, 5, 6-dibenzanthracene, 20-methylcholanthrene, and 2-amino-5-azotoluene, it is possible that other compounds may induce pulmonary tumors more readily in some strain of mice other than strain A.

In any case, constant control groups for comparison of the incidence of pulmonary tumors with that in the experimental groups are essential. When weak carcinogens and low doses of potent carcinogens are used, conclusive results are difficult to obtain with this procedure. It can be stated, however, that any compound with which multiple tumors of the lung are obtained in more than 50 percent of strain A mice 3 months after injection (the mice being 6 months old at the time) is positive in having elicited these neoplasms.

In a previous report (*31*), certain factors which influence the biologic testing of carcinogens by the subcutaneous-injection technique were summarized. In another report (*12*), some of the factors influencing the induction of pulmonary tumors were outlined. The dose, the route of injection, the medium in which the chemicals are introduced, and the time after administration were emphasized.

The importance of these factors is stressed again in the present paper. In the first place, the results obtained in the lungs when the chemical is introduced intravenously cannot be compared, without reservation, with the findings after subcutaneous injection. It appears that while 20-methylcholanthrene is more potent than 1,2,5,6-dibenzanthracene intravenously, it is less potent when injected subcutaneously as an oily solution. The carcinogenicity for the lungs of both can be sharply limited if the chemicals are held at the site of injection by adsorption on charcoal (*32*).

Although the number of pulmonary tumors is closely proportional to the dose of the carcinogen injected, the observations are limited to 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene in 0.1- to 1.5-mg. doses. It is probable that there is a critical dose below which no pulmonary tumors are obtained. These results are indicated by Lettinga (*33*), who found an abrupt transition from a small to a large number of pulmonary tumors when the subcutaneous dose of 1,2,5,6-dibenzanthracene in lard was increased from 5×0.2 mg. to 5×0.5 mg., and suggest an overflow of the carcinogen from the subcutaneous site of injection as the cause of the pulmonary tumor genesis (*34*). At high doses, there is probably an amount above which no further increase in the number of pulmonary tumors can be obtained, comparable to the maximum effective dose for the induction of subcutaneous tumors (*35*).

The subcutaneous route of injection is not so desirable as the intravenous for the evaluation of the carcinogenicity of compounds by the pulmonary-tumor-induction method. Fewer and later tumors are obtained, and the carcinogenicity of the compounds is probably modified by the ability of the agents to dissolve and to reach the lungs without destruction or conjugation, which may alter its carcinogenic potency. These characteristics may or may not be correlated to the carcinogenic potency of the chemical. On the other hand, it is quite possible that some compounds may be effective in inducing pulmonary neoplasia when injected sub-

cutaneously and ineffective intravenously, owing to their rapid alteration and elimination from the blood stream.

The pulmonary neoplastic reaction is an important adjunct to the subcutaneous testing of carcinogens when susceptible mice, such as strain A or C, are used. Some compounds, such as 1,2,7,8-dibenzacridine, have given negative results in that subcutaneous sarcomas were not elicited (*20*) at the site of injection, yet the compound produced multiple pulmonary tumors. The examination of the lungs of the mice thus furnishes a simultaneous and an additional test medium for the evaluation of the compounds.

The results obtained by any one biologic evaluation of a carcinogen will not necessarily agree with the results obtained by the use of a different method. No single test method, therefore, is adequate for the evaluation of the compounds. Generally the subcutaneous-injection technique and the skin-painting procedure produce results in close agreement, although occasional but notable exceptions are encountered. Thus Shear reported that 10-methyl-1,2-benzanthracene (*36*) and 1′2′-dihydro-4′-methyl-3,4-benzpyrene (*18*) are fairly potent carcinogens when injected subcutaneously, but are weak when the skin-painting technique is used. Conversely, 2-methyl-3,4-benzphenanthrene is more potent to the skin than to the subcutaneous tissues.

The pulmonary-tumor-induction technique, however, produces results which in many instances are strikingly different from those obtained by the subcutaneous or percutaneous methods of testing. Whereas 3,4-benzpyrene is the most potent carcinogen, after 20-methylcholanthrene, by both the subcutaneous and skin-painting tests (*23, 24*), it is a relatively weak evoker of pulmonary tumors; 1,2,5,6-dibenzanthracene, on the other hand, is less effective in its subcutaneous and percutaneous potency than 3,4-benzpyrene and 3,4,5,6-dibenzcarbazole, yet its ability to induce pulmonary tumors is much higher than that of the two compounds; and although 15-16-benzdehydrocholanthrene is reported to be a very weak carcinogen subcutaneously (*17*), it is quite active in inducing pulmonary tumors by the intravenous route.

All of these compounds, however, if positive by the skin-painting or subcutaneous-injection tests, also induce pulmonary tumors, and vice versa. The 2-amino-5-azotoluene, which is not capable of producing subcutaneous sarcoma

TABLE 6.—*Relative carcinogenic potency of thirteen compounds for the skin, subcutaneous tissue, and the lungs of mice*

Compound	Subcutaneous injection	Percutaneous application	Pulmonary-tumor induction by—	
			Intravenous injection	Subcutaneous injection
20-methylcholanthrene	High	High	High	High.
3,4-benzpyrene	do	do	Medium	Low.
3,4,5,6-dibenzcarbazole	Medium	Medium	do	Medium.
1,2,5,6-dibenzanthracene	do	do	High	High.
4′-methyl-3,4-benzpyrene	do		Low	?Low.
2-methyl-3,4-benzphenanthrene	Low	Medium	do	Low.
15,16-benzdehydrocholanthrene	do		Medium	Do.
1,2,5,6-dibenzacridine	?Low	Low	do	High.
1,2,7,8-dibenzacridine	?Negative	do		Medium.
3-methoxy-10-propyl-2-benzanthracene	Negative		Negative	Negative.
1,2-benzanthracene	do	Negative	do	Do.
2-amino-5-azotoluene	do	do		Positive.
Thorium dioxide	Positive		Negative	Negative.

or skin carcinoma in mice, does induce pulmonary tumors. Conversely, whereas 0.2 cc. of a 25-percent colloidal suspension of thorium dioxide [6] has induced a few subcutaneous sarcomas at the site of injection, three and four times the amount, given intravenously in several doses, has failed to elicit pulmonary tumors in strain A mice.

Some of the discrepancies encountered in the determinations of the carcinogenic potency of a few chemicals tested by the subcutaneous-injection, skin-painting, and pulmonary-tumor-induction methods are summarized in table 6.

The findings demonstrate that certain compounds are specific in their effect on certain tissues. The relative nature of the concept of carcinogenicity is emphasized, as well as the fact that the results of experiments on one tissue of one species of animal cannot be applied to other tissues of the same animal, much less to other species.

[6] *Thorotrast, Heyden Chemical Corporation. Full details of the investigation of this preparation will be presented at a later date.*

SUMMARY

The induction of pulmonary tumors by the intravenous injection of aqueous suspensions of compounds into strain A mice can be used as a quantitative test for the carcinogenic potency of a wide variety of chemicals.

The following compounds have been found to produce pulmonary tumors in strain A mice after intravenous or subcutaneous injection: 20-methylcholanthrene; 1,2,5,6-dibenzanthracene; 3,4,5,6-dibenzcarbazole; 3,4-benzpyrene; 15,16-benzdehydrocholanthrene; 1,2,5,6-dibenzacridine; 1,2,7,8-dibenzacridine; 2-methyl-3, 4 benzphenanthrene; 4′-methyl-3, 4-benzpyrene; and 2-amino-5-azotoluene.

The following compounds did not induce pulmonary tumors in strain A mice: 1, 2 benzanthracene; 3-methoxy-10-propyl-1, 2-benzanthracene; and colloidal thorium dioxide.

There is no constant quantitative or qualitative correlation between the carcinogenicity of a compound for the lungs and for the subcutaneous or the cutaneous tissues of mice.

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INDUCED PULMONARY TUMORS IN MICE. III. THE ROLE OF CHRONIC IRRITATION IN THE PRODUCTION OF PULMONARY TUMORS IN STRAIN A MICE

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The role of irritation and inflammation in the process of carcinogenesis with cancer-provoking aromatic polycyclic hydrocarbons remains unelucidated. It is agreed that "chronic irritation" is an inadequate term to describe the neoplastic reaction after the introduction of carcinogens into animal tissues (*1*), and the direct causal relationship between irritation and inflammation and carcinogenesis with the carcinogenic hydrocarbons is no longer generally maintained. Beck (*2*) concluded that acute and subacute inflammation does not influence the carcinogenicity of 3,4-benzpyrene in mice and that the inflammatory fibrous tissue neither retards nor accelerates the development of sarcoma which does not originate from the fibrous tissue. Page (*3*) stated that there is no evidence that an inflammatory reaction plays any part in the carcinogenic action of benzene solutions of 20-methylcholanthrene or cholanthrene applied to the skin of mice.

On the other hand, some investigators feel that irritation and inflammation are at least indirectly involved in the process of carcinogenesis. Campbell (*4–8*), in his extensive work on the effect of dusts and exhaust gases upon the incidence of pulmonary tumors in mice, reported that inorganic constituents such as silica and iron enhance or aid the effects of the specific carcinogens in the dust from tarred roads, and concluded (*8*) that "irritation without inflammation appears to play a part in the genesis of these tumors." Orr (*9*) postulated that carcinogenic agents in pellets introduced subcutaneously into mice prevent the occurrence of an adequate fibrous reaction and that sarcoma may be the result of such persistently incomplete reaction.

The following experiments, begun in September 1938, were undertaken to study the effect of nonspecific irritation on the incidence of primary pulmonary tumors in mice, and the role that inflammation and irritation may play in the induction of pulmonary tumors with carcinogenic hydrocarbons.

Concurrently, an experiment with soot was designed as a preliminary evaluation of test methods involved in the systematic investigation of the carcinogenic properties of various atmospheric dusts, a project now under way at the National Cancer Institute. Campbell's experiments (*4–8*) have indicated that this type of survey may be of public health significance.

EXPERIMENTAL PROCEDURE

In published accounts (*4–8*, *10*, *11*) of the effect of dusts upon the incidence of primary pulmonary tumors in mice the inhalation technique of introducing the substances into the lungs has been used, in which the animals are kept in dust-laden containers which are stirred periodically. This method has the advantage of maintaining the normal conditions of introducing the foreign material into the lungs. The amount of the substance inhaled, however, cannot be determined readily; moreover, the whole animal and not its respiratory tract only is exposed to the agent. More exact conditions can be

obtained with special inhalation chambers, such as designed by Lorenz (*12*), but they are complicated and require constant attention.

Foreign material also can be introduced into the lungs of animals by means of intratracheal injection. It is possible to inject about 0.1 cc. of an aqueous suspension into the tracheae of mice, but the tediousness of the operation, the high mortality, and the small amount of the substance which can be introduced makes this method unsatisfactory (*13*).

Mice, however, can be injected intravenously easily and quickly and tolerate surprising amounts of suspended material which lodges in the lungs. The dose of the injected material can be determined with accuracy and repeated doses administered. It was therefore decided to use the intravenous route of injection in studying the effect of various substances on the incidence of pulmonary tumors in mice.

Strain A mice, which are extremely susceptible to spontaneous and to induced primary pulmonary tumors (*14*, *15*), were chosen as the experimental animals. The mice were obtained from the Roscoe B. Jackson Memorial Laboratory of Bar Harbor, Maine. They were kept under the same environmental conditions and received a diet of Purina dog-chow pellets and an unlimited supply of water. Equal numbers of males and females were used in each group, and the sexes were kept separate. All animals were injected when they were from 2 to 3 months of age.

Previous investigations (*16*, *17*) determined that primary pulmonary tumors of mice are easily diagnosed in the gross, especially after fixation in Tellyesniczky's fluid. Since the great majority of these discrete, white, round pearly nodules occur immediately underneath the pleura, a good approximation of their number can be obtained by counting the nodules visible on the external surfaces of the organ. Grossly and histologically, the induced tumors are identical with those of spontaneous origin. The main criteria for determining whether the pulmonary tumors are induced or spontaneous are the incidence of the tumors in the experimental group as compared with the control animals of the same age, and the number of individual pulmonary tumors in each animal. Spontaneous pulmonary tumors in strain A mice under 9 months of age are almost always single, whereas the tumors induced with the carcinogenic hydrocarbons are multiple. The average number of induced pulmonary tumors is directly proportional to the dose of the carcinogen.

The experiments were not prolonged over 6 months since at that time the mice were 8 or 9 months old. Above this age the incidence of spontaneous pulmonary tumors is sufficiently high to confuse the results (*16*, *17*).

For subcutaneous injection of soot extracts, male mice of strain C_3H, obtained from the Jackson Laboratory, were used. They were maintained under the same conditions and were injected at the same age as the strain A animals.

Single or semi-serial sections were taken of the lungs of strain A mice and of the tumors induced in the C_3H mice. The sections were stained with haematoxylin and eosin for histologic examination, for which we are indebted to Dr. Hugh G. Grady of this laboratory.

EXPERIMENT 1

Five samples of ore were procured from the Geological Museum of Harvard University. According to Mellor (*18*), the constituents of the ores are approximately as follows:

(1) Arsenopyrite (mispickel)—FeAsS, with 46 percent inorganic arsenic.

(2) Chromite—Cr_2O_3, 39–60 percent; FeO, 13–25 percent; MgO, 5–16 percent; Al_2O_3, 3–17 percent; SiO_2, 1–8 percent.
(3) Quartz—SiO_2.
(4) Thorite—ThO_2, 48–70 percent; SiO_2, 14–19 percent; U_2O_3, 0.5–9 percent; Fe_2O_3, 0.3–8 percent; PbO, 0.4–2 percent; CaO, 0.4–3 percent.
(5) Uraninite (pitchblende or nasturan)—U_3O_8 with some lead uranate.

The ores were pulverized in a mechanical mortar so that the average of the particles obtained was 3.5 micra for arsenopyrite, 2.0 micra for uraninite, and 1.6 micra each for chromite, quartz, and thorite. The powders were suspended in isotonic saline, 1.0 gm. per 100 cc., and sterilized by heating.

Strain A mice, about 50 for each ore, were given a single injection of the suspension into the lateral tail vein. The mice tolerated 0.5 cc. (5 mg.) of the suspensions with about a 25-percent immediate morality; with quartz, however, the dose had to be reduced to 0.1 cc. (1 mg.). A group of 75 mice of the same age was kept as untreated controls.

From 10 to 20 mice were killed at 3, 4.5, and 6 months after injection, and at the same time one-third of the controls were killed. The lungs were examined for the presence and the number of pulmonary nodules.

Results

The mice injected with uraninite died of uranium nephritis within a few weeks after the administration of the ore. When the same mortality was found in mice injected with 0.4 mg. of uraninite, the ore was abandoned in further experiments. Only 3 out of 20 mice survived for 6 months with the latter dose. All other animals survived the term of the experiment if they recovered from the initial injection procedure.

In the majority of the mice, the injected ore was evident on gross inspection of the lungs.

Histologically, there was no qualitative difference in the reaction of the lungs to the various ores, whether the animal was killed 3, 4.5, or 6 months after injection. The greatest amount of reaction was encountered in the lungs of mice injected with arsenopyrite or quartz, and much less in the animals which had received chromite or thorite.

Particles of the ores were visible in all sections of the lung, although there was a great variability in the amount of the ore deposited. With quartz, most of the particles were invisible until observed under polarized light. The smaller particles lay in the alveolar walls, while the larger ones or clumps of small particles were seen plugging small blood vessels.

Acute inflammation, as evidenced by the presence of a few polymorphonuclear leukocytes, was present around one particle of chromite. In the rest, two types of reaction were present. Most common were small nodules, mostly subpleural, consisting of nests of small lymphocytes with occasional large cells which appeared to be macrophages (fig. 1). The ore particles were seen lying free on the periphery or in the center of these nodules; much smaller particles were phagocytosed. The second type of reaction appeared in the form of similar-sized nodules consisting of a small number of lymphoid cells and a larger number of macrophages with large, irregular nuclei and containing small phagocytosed granules in the cytoplasm (fig. 2). This reaction resembled an early silicotic nodule. Proliferation of the alveolar lining cells was seen around the particles of the ores lying in those structures, but no pulmonary fibrosis was present in any of the sections. It is emphasized that the histologic evidence of chronic inflammation was minimal in all sections.

As summarized in table 1, there was no increase in the incidence or the number of

FIGURE 1.—Reaction of lung to intravenously injected arsenopyrite, 4.5 months after injection. Subpleural lymphocytic nodule on the right, smaller nodule with lymphocytes, macrophages, and clumps of ore particles on the left. Haematoxylin and eosin. × 335.

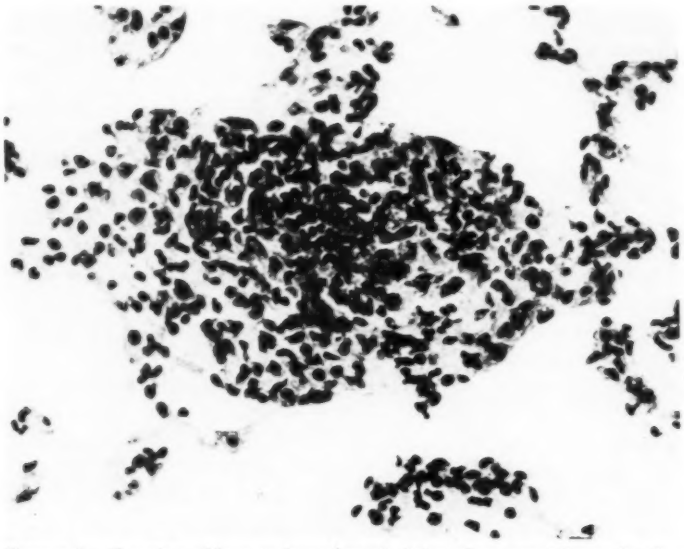

FIGURE 2.—Reaction of lung to intravenously injected quartz, 4.5 months after injection. Small nodule composed of lymphocytes and macrophages, resembling an early silicotic nodule. Quartz particles were visible under polarized light. Haematoxylin and eosin. × 435.

pulmonary tumors in individual animals in the mice injected with the ores as compared with the controls. The data are recapitulated on semi-logarithmic paper in the graph (fig. 3, lines *A* and *B*), combining the mice treated with all four ores into one group. The points on the graph are derived from the carcinogenic index of Iball (*19*) and applied to the pulmonary tumors in mice (*16*, *17*): the percent of mice developing pulmonary tumors is multiplied by the average number of pulmonary tumors per positive animal, the time being constant.

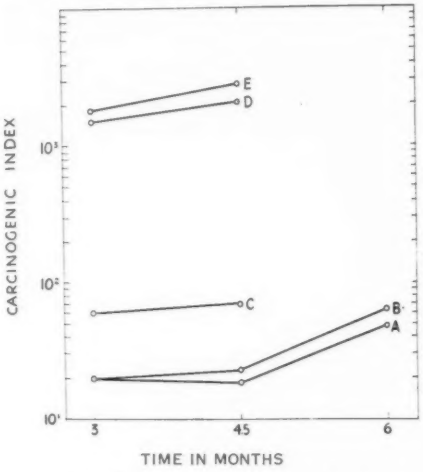

FIGURE 3.—Response of lungs of strain A mice to intravenously injected ores and soot. *A*, Single injection of 5.0 mg. of arsenopyrite, chromite, or thorite, or 1.0 mg. of quartz. *B*, Untreated controls. *C*, Single injection of 2.5 mg. of unextracted soot. *D*, Injection of ores as in *A*, 0.1 mg. of methylcholanthrene a week later. *E*, Single injection of 0.1 mg. of methylcholanthrene. Carcinogenic index—percent of mice developing pulmonary tumors times average number of tumors per positive animal.

The incidence of pulmonary tumors in mice 3 months after injection, 20 percent, is somewhat higher than the incidence of spontaneous tumors determined previously, 10 percent (*16*, *17*). The lack of difference between the controls and the experimental mice, however, indicates that this variation is not significant. It also shows that with the lack of a clear end point between induced and spontaneous pulmonary tumors in mice, constant controls for comparison are essential.

It is concluded that with a single intravenous injection of 5 mg. of four metallic ores of an average particle size of 1.6 to 3.5 micra, no lung tumors were induced in strain A mice despite the presence of irritation. Within the limits of the experimental procedure, therefore, this is further evidence that chronic irritation *per se* does not produce pulmonary neoplasms in mice.

TABLE 1.—*Effect on pulmonary-tumor incidence in strain A mice of single intravenous injection of various ore dusts suspended in saline*

Substance	Dose	Period after injection when animals were killed								
		3 months			4.5 months			6 months		
		Number of mice	With lung tumors	Average number of lung tumors	Number of mice	With lung tumors	Average number of lung tumors	Number of mice	With lung tumors	Average number of lung tumors
	Mg.									
Arsenopyrite	5	15	2	1	15	2	1	12	5	1.1
Chromite	5	10	3	1	10	3	1	17	9	1.2
Quartz	1	11	3	1	10	1	1	20	8	1.2
Thorite	5	15	2	1	14	2	1	20	4	1.5
Uraninite	0.4							3	1	1.0
Controls		25	5	1	25	6	1	22	9	1.3

EXPERIMENT 2

In order to ascertain whether chronic irritation would influence the induction of pulmonary tumors by a carcinogenic agent, groups of 20 strain A mice were injected intravenously concurrently and with the same amounts of arsenopyrite, chromite, quartz, and thorite as in experiment 1. Another group was injected with 2.5 mg. of soot which had been extracted with benzene, in 0.25 cc. of saline.

A week after the intravenous injection of the ores or the benzene-extracted soot, all mice were given a single intravenous injection of 0.1 mg. of 20-methylcholanthrene dispersed in 0.25 cc. of horse serum saturated with cholesterol.[1] Another group of 30 strain A mice, previously untreated, was injected with the same dose of the hydrocarbon for comparison.

Half of the animals were killed at 3 months, and half at 4.5 months after the administration of methylcholanthrene, and the lungs examined for the presence and the number of pulmonary tumors.

[1] *Prepared by Lorenz (20). The hydrocarbon was a synthetic product with a melting point of 179.8°–180.4° C. (corr.).*

RESULTS

The histologic sections of the lungs of mice injected with ores and with methylcholanthrene showed reactions identical with those described in mice which had received the ore alone, except for the presence of multiple primary pulmonary tumors. There was no correlation between the sites of inflammatory reaction and the pulmonary tumors (fig. 4), although in several sections the inflammatory lymphocytic nodules occurred near or within the tumors (fig. 5). Neither

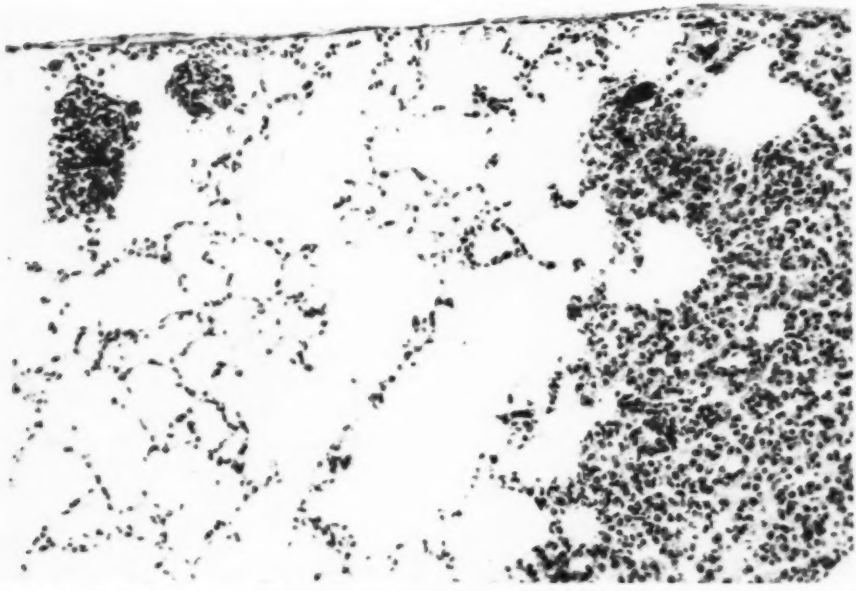

FIGURE 4.—Reaction of lung to intravenously injected arsenopyrite, followed by 0.1 mg. of methylcholanthrene 1 week later. Two inflammatory lymphocytic nodules on the left, and one on the right containing ore particles, at the edge of a pulmonary tumor. Haematoxylin and eosin. × 155.

TABLE 2.—*Effect of single intravenous injection of various ore dusts upon pulmonary tumors in strain A mice induced with 0.1 mg. of 20-methylcholanthrene intravenously*

Substance	Dose	Period after injection when animals were killed					
		3 months			4.5 months		
		Number of mice	With lung tumors	Average number of lung tumors	Number of mice	With lung tumors	Average number of lung tumors
	Mg.		*Number*			*Number*	
Arsenopyrite	5	10	10	8.0	10	10	12.1
Chromite	5	9	9	11.2	9	9	20.8
Quartz	1	10	10	18.1	8	8	22.0
Thorite	5	10	10	15.2	10	10	22.1
Soot extracted with benzene	2.5	10	10	15.8	10	10	16.6
		15	15	18.2	14	14	27.8

did the lungs which had the greatest amount of inflammatory reaction contain the largest number of neoplastic nodules.

The primary pulmonary tumors were identical with the tumors described previously (*16*, *17*). They were adenomatous tumors of uniform appearance, situated just below the pleura, and consisted of moderately large round or cuboidal cells with definite boundaries and large round

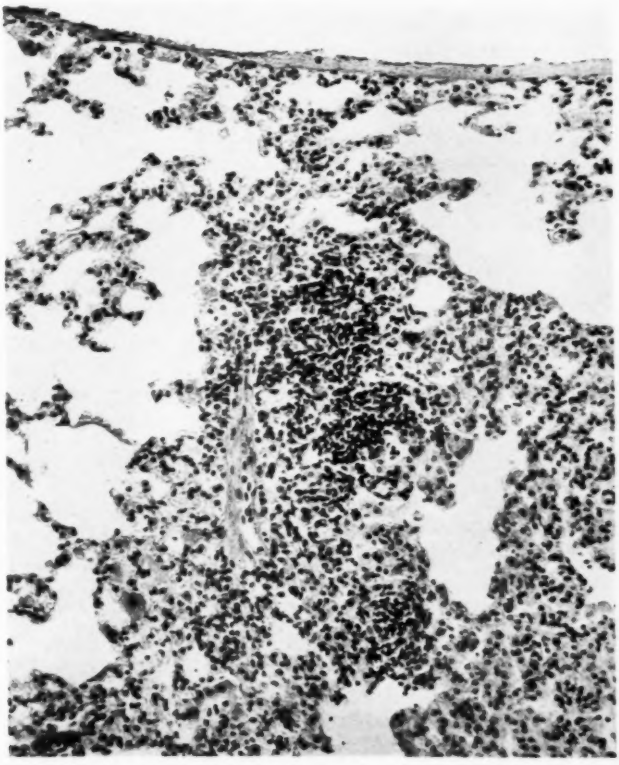

FIGURE 5.—Reaction of lung to intravenously injected quartz, followed by 0.1 mg. of methylcholanthrene 1 week later. The inflammatory nodule, in which particles of quartz were visible under polarized light, is surrounded by multinucleated giant cells and by the pulmonary tumor. Haematoxylin and eosin. × 150.

or oval nuclei. The tumors were not encapsulated, the growth was fairly solid, and many of the cells were arranged around a central cavity suggesting alveolar arrangement (figs. 4 and 5).

As summarized in table 2 and in the graph (fig. 3, lines *D* and *E*), there was no appreciable difference between the number of pulmonary tumors in mice which had received the ores or the benzene-extracted soot and the methylcholanthrene injection, and the mice which had not been injected previously with ores. In a previous series with 0.1 mg. of methylcholanthrene, the number of pulmonary tumors at 3 and 4.5 months was somewhat lower than in the present groups, so that the slightly smaller number of tumors in the mice which had received the ores previous to the hydrocarbon is well within the range of experimental error.

It is concluded that the presence of nonspecific irritants, within the dose, particle size, and time used in these experiments does not influence the appearance and the number of pulmonary tumors induced with intravenously injected methylcholanthrene.

EXPERIMENT 3

To determine the methods suitable for detecting the presence of carcinogens in atmospheric dusts, a sample of soot was collected from the base of a chimney burning soft coal.

The soot was extracted with benzene in a Soxhlet apparatus, and also by the quicklime-ether method used by Passey (*21*). The soot was mixed with quicklime and water into a paste, spread out to dry, and then extracted with ethyl ether in a Soxhlet apparatus.

From 500 gm. of soot, 3.39 gm. of tarry material were extracted with benzene; and 2.21 gm. were extracted from 1,000 gm. of soot by the quicklime-ether method. The extracts were mixed with lard, filtered at 37° C., in which they were partially soluble, in concentrations of 8 percent for the benzene extract, and 6 percent for the quicklime-ether extract of soot. Thus, 1 cc. of the lard mixture represented the material obtained from 11.9 gm. of soot for the benzene extract, and 27.1 gm. of soot for the ether extract.

The mixtures were injected subcutaneously into the right axilla of C_3H strain male mice. About half of the animals received a single injection of 0.25 cc., while the other half were injected every 2 weeks with 0.1 cc. for a total of 10 injections. Twenty C_3H mice were injected subcutaneously 10 times with 0.1 cc. of lard as controls.

Thirty strain A mice also received the same amount of soot extracts subcutaneously, half being given a single injection and half repeated injections.

At the same time, the unextracted soot and the soot which had been extracted with benzene were suspended in sterile isotonic saline solution, 1 gm. per 100 cc., and 0.25 cc. of the suspensions was injected intravenously into strain A mice. Thirty animals received the unextracted soot, and 45 the soot which had been extracted with benzene. The average particle size of the unextracted soot was 2.4 micra, and of the benzene-extracted soot, 1.8 micra.

The subcutaneously injected animals were examined weekly for the presence of tumor at the site of injection. The intravenously injected mice were killed at 3, 4.5, and 6 months after injection and their lungs were examined for the presence and number of pulmonary nodules.

RESULTS

All of the strain A mice which were injected subcutaneously with the extracts developed large ulcers at the site, even when a single injection was given, and all had to be killed 5 months after the experi-

TABLE 3.—*Subcutaneous injection of extracts of soot into strain C_3H males*

Material	Concentration in lard	Equivalent weight of original sample	Dose	Mice	Number of tumors and time (in months)										Total tumors	Average time
					6	7	8	9	10	11	12	13	14	15		
	Percent	*Grams*	*Cc.*	*No.*											*No.*	*Months*
Soot-benzene extract	8	2.97	0.25	5			1	1			1				3	9.7
Do	8	11.9	[1] 1.0	5					1					1	2	12.5
Soot-quicklime-ether extract	6	6.77	.25	12	1	2				1				1	5	7.8
Do	6	27.1	[1] 1.0	10		1	1			1					3	8.7
Lard			[1] 1.0	19												

[1] Total dose: single dose was 0.1, administered 10 times.

mental procedure. There were no local tumors, and the incidence of pulmonary tumors was obscured by the presence of extensive chronic pneumonic infiltration.

Slight ulceration occurred in the C_3H mice which received repeated injections of the extracts, but all of the mice survived the procedure without undue impairment of health. Six months after the initial injection, tumors began to appear and in 15 months, 13 spindle-cell sarcomas were obtained, as summarized in table 3.

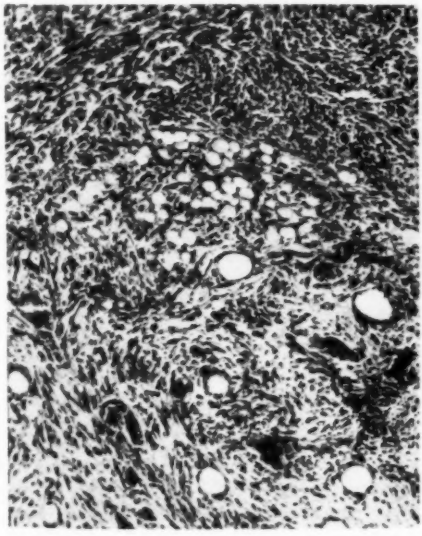

FIGURE 6.—Spindle-cell sarcoma in C_3H male mouse, at the site of repeated subcutaneous injections of benzene extract of soot. Haematoxylin and eosin. × 130.

Transplantation of a tumor into other C_3H mice was performed in one instance, with a tumor arising after injection with the ether extract of soot. The tumor grew successfully and was discontinued after one passage.

The histologic appearance of these tumors was the usual spindle-cell sarcoma obtained with carcinogenic hydrocarbons (fig. 6). One tumor, evoked with the ether extract of soot, was a mixed myofibrosarcoma.

No other tumors were seen in the C_3H mice until the fifteenth month after injection, when pulmonary tumors and hepatomas began to appear. The experiment was terminated 17 months after the original injections, or when the mice were about 20 months of age. At that time, 15 mice injected with the soot extracts and 9 mice injected with lard were killed. Of the 16 which received the extracts, 8 had hepatomas and 9 had pulmonary tumors; 1 had 5 pulmonary nodules, and 8 had single tumors of the lung. Of the 9 lard-injected controls, 3 had hepatomas, and no pulmonary tumors were seen.

C_3H male mice over 15 months old of a colony maintained at this laboratory have a spontaneous incidence of 40 percent hepatomas (*22*). The occurrence of hepatomas in the lard-injected controls indicates that these tumors were of spontaneous origin in both groups. The incidence of spontaneous pulmonary tumors in C_3H

males over 15 months of age, however, is about 8 percent (*22*), and it is felt that the incidence of 60 percent in the animals injected with the soot extracts represents an induction of these neoplasms by the injected material.

The results in strain A mice which received a single intravenous injection of unextracted soot, or soot which had been extracted with benzene are summarized in table 4. It is evident that the soot extracted with benzene did not increase the incidence of pulmonary tumors in the animals. With the unextracted soot, on

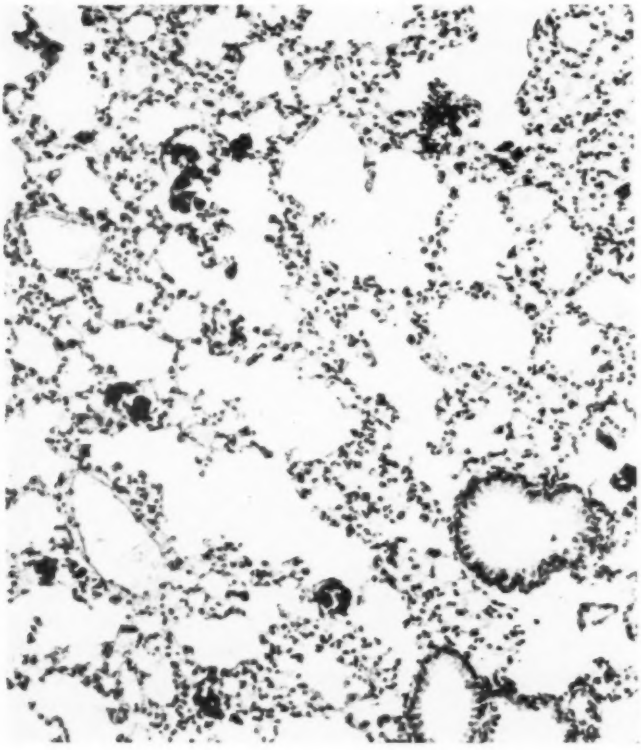

FIGURE 7.—Reaction of lung to soot injected intravenously, 4.5 months after injection. Note the distribution of the particles and the minimal inflammatory reaction. Haematoxylin and eosin. × 120.

TABLE 4.—*Effect of unextracted soot and soot extracted with benzene, injected as saline suspension intravenously, upon pulmonary tumor incidence in strain A mice*

Substance	Dose	Period after injection when animals were killed								
		3 months			4.5 months			6 months		
		Number of mice	With lung tumors	Average number of tumors	Number of mice	With lung tumors	Average number of tumors	Number of mice	With lung tumors	Average number of tumors
Soot extracted with benzene	*Mg.* 2.5	14	2	1	14	2	1	15	2	1
Soot, unextracted	2.5	15	6	1.5	15	8	1.3			
Controls		22	5	1	25	6	1	22	9	1.3

the other hand, the incidence of pulmonary tumors was twice as high as in the untreated controls and in the mice injected with the benzene-extracted soot. Moreover, whereas more than one pulmonary nodule was not seen in the two latter groups of mice killed before 6 months after the experimental procedure, three mice injected with soot and killed 3 months after the injection had two individual pulmonary tumors, and at 4.5 months after injection two out of eight mice had two pulmonary tumors each. The carcinogenic index values for the incidence and number of pulmonary tumors in the mice injected with the unextracted soot are given in figure 3, line *C*.

The pulmonary tumors were of the same histologic appearance as those described previously. Although particles of the soot (fig. 7) were present near or within some of the tumors, there was no correlation between the areas of tumor invasion and the sites of heaviest soot deposits. That the site of tumor origin apparently does not correspond to the location of the highest concentration of the introduced carcinogen, as evidenced by the charcoal particles to which 1,2,5,6-dibenzanthracene is adsorbed, was reported previously (*13*). The inflammatory reaction of the lungs to the extracted and the unextracted soot was minimal, being less than with any of the ores in experiment 1.

It is felt that pulmonary tumors were induced in strain A mice following the single intravenous injection of 2.5 mg. of this sample of unextracted soot. One of the great deficiencies of this method of biologic testing is the lack of a clear end point; i. e., the control animals develop pulmonary tumors as well as the experimental ones, so that conclusions can be reached only through the quantitative differences in the two groups. This is also evident in Campbell's work (*4–8*). The objection cannot be overcome by the use of mice more resistant to spontaneous pulmonary tumors, because all strains develop the neoplasms if they are permitted to live to a sufficient length of time (*16, 17*). The use of more resistant strains would necessitate the use of larger amounts of the substances to be tested by the intravenous route of injection. In these experiments, however, only a single injection was given, and the amount was limited by the tolerance of the animals to the dose. Much more definite results probably can be obtained if the injections are repeated weekly or every 2 weeks for several doses. One of the main advantages of the method is that suggestive results, which are confirmed by the subcutaneous-injection technique, can be obtained within 3 months after injection.

DISCUSSION

The incidence and the number of primary pulmonary tumors in mice of susceptible strains can be increased by the introduction into the animal of carcinogenic hydrocarbons and related compounds. The susceptibility of the mice to induced pulmonary tumors is parallel to their susceptibility to spontaneous tumors of the lung, i. e., strains which are most susceptible to their spontaneous occurrence are most susceptible to their induction with carcinogens. The carcinogenic chemicals can be injected by various routes, including the intratracheal, in order to elicit the neoplastic response in the lungs.

Experiments conducted at this laboratory (*12, 13*) suggest that the increase in the number of pulmonary tumors in mice exposed to certain dusts is not owing to nonspecific irritation but to the presence of some carcinogenic material in the dust. Campbell (*4–8*) obtained the highest incidence of pulmonary tumors in mice exposed to dust from tarred roads. It is known that some coal tars contain a power-

ful carcinogenic agent, 3,4-benzpyrene. The increase in the incidence reported with dust which had been extracted with benzene was not so impressive, and no indication concerning the completeness of the extraction was given. Contrary to Campbell's postulation that nonspecific irritation plays a part in the genesis of pulmonary tumors in mice, the experiments reported here fail to indicate that nonspecific irritation produces pulmonary tumors in mice or aids in their induction with carcinogens. It is also significant that the development of pulmonary tumors in mice of strain A injected subcutaneously or intravenously with 1,2,5,6-dibenzanthracene or 20-methylcholanthrene is not associated with any demonstrable inflammatory reaction (*21*).

One of the possibilities given in explanation of the increase in the pulmonary tumors in mice painted with tar is that the procedure in some way alters the physical state of the animal so that its resistance to carcinogenesis is lowered, and pulmonary tumors arise at points of incidental irritation of inhaled dust particles (*25*). Mice born and raised in an atmosphere practically devoid of dust are just as susceptible to the induction of pulmonary tumors after the subcutaneous injection of 1, 2, 5, 6-dibenzanthracene as are the animals maintained under normal conditions (*12*). The present report also supports the view that no external adjuncts, such as atmospheric dust, appear to be needed in the production of pulmonary tumors in mice with carcinogenic agents.

Whether the action of carcinogens, such as 20-methylcholanthrene, is a general one, producing tumors secondary to some alteration in the whole organism, or whether its action is a local one upon the tissues at the site of application, remains one of the most important undetermined questions. The present available evidence, however, points toward local action in the induction of pulmonary tumors (*23*). After the subcutaneous injection of 1, 2, 5, 6-dibenzanthracene the absorption spectrum fails to reveal the presence of the compound in the lungs,[2] although pulmonary tumors are induced. That the agent or some active derivative does reach the lungs, however, is suggested by the presence of photodynamic activity of emulsons of the lungs of mice injected subcutaneously with 3,4-benzpyrene (*26*).

The increase in the incidence of bronchogenic pulmonary carcinoma in man has attracted the attention of oncologists and public health officials. One of the theories presented is that the stimulus for its occurrence may be found in the atmospheric dust particles introduced into the lungs. Passey (*21*) in 1922 demonstrated the presence of compounds carcinogenic to mice in the soot of soft coal discharged into the air Campbell (*4–8*) established that there was a marked increase in the incidence of primary pulmonary tumors in mice exposed to the dust of tarred roads.

The presence of active carcinogens in the atmospheric dusts may be of public health significance and deserves systematic investigation and attention. This report indicates the biologic testing which may be applied for the project: (1) The subcutaneous injection into male mice of strain C_3H of benzene and other extracts of dusts gathered in various localities; and (2) the intravenous injection of the unextracted dusts into mice of strain A.

It must be remembered, however, that experiments on mice should not be applied, except as leads for further investigations, to other species of animals, including man. Thus, although 20-methylcholanthrene and 1, 2, 5, 6-dibenzanthracene in 0.1 mg. intratracheal doses induced pulmonary tumors in strain A mice (*13*), the

[2] *Lorenz, E.: Unpublished data.*

introduction of much larger amounts into the trachea of rats failed to produce tumors of the lung in these animals (*27*). The only authoritative reports of the induction of pulmonary neoplasms in man by means of inhaled dust are those of the Schneeberg and Joachimstahl miners (*28*) who were exposed to radioactive ores. Whether substances which are carcinogenic to mice are also carcinogenic to man, and in what doses and under what conditions, is a matter of conjecture. The pulmonary tumors in mice are similar to the pulmonary neoplasms of man in that both are found in the lungs. But, whereas the human neoplasm is believed to be almost exclusively a bronchogenic carcinoma, the mouse tumor is an adenoma or adenocarcinoma arising from the alveolar wall lining, and quite different in its development, site, and biology.

SUMMARY

The single intravenous injection of 5 mg. of arsenopyrite, chromite, or thorite, or of 1 mg. of quartz ore (particle size 1.6 to 3.5 micra) did not induce primary pulmonary tumors in strain A mice within 6 months after the administration, despite the presence of chronic irritation.

The intravenous injection of these ores did not increase the number of primary pulmonary tumors, nor did it apparently have any other effect upon the development of such tumors following the intravenous administration of 0.1 mg. of 20-methylcholanthrene.

Soot from a chimney burning soft coal contains a benzene-soluble compound capable of initiating subcutaneous sarcoma and primary pulmonary tumors in strain C_3H male mice. The single intravenous injection of 2.5 mg. of the unextracted soot increased the incidence of primary pulmonary tumors in strain A mice.

Acknowledgment is made of the technical assistance of Mr. Walter F. Gately.

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THE ACTION OF 2-AMINO-5-AZOTOLUENE IN THE PRODUCTION OF LIVER TUMORS OF RATS AND THE BEHAVIOR OF THESE TUMORS IN VITRO

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INTRODUCTION

The production of tumors of the liver by the oral administration of the aniline dye 2-amino-5-azotoluene was first demonstrated by Yoshida in 1932 (*1*). This work was confirmed and extended by the later work of Yoshida (*2*), by Sasaki and Yoshida (*3*), Zylberszac (*4*) and by Waters (*5*). Sasaki and Yoshida (*3*) found for rats, and Shear (*6*) for mice that tumors were produced in the livers and even though the amino-azotoluene was injected subcutaneously no tumors appeared at the injection site. Similar tumor-producing activities were found for other closely related chemical substances by Maruya (*7*), Harada (*8*), and by Kinosita (*9*).

During the last several years work has been in progress in this laboratory on the mode of action of 2-amino-5-azotoluene with respect to its carcinogenic action on the livers of rats. This work necessitated first the production of lesions in the livers of rats through the feeding of the dye substance to the experimental animals. The study then involved an attempt to culture and examine the epithelial cells of these lesions and to correlate the growth *in vitro* and behavior of these cells with the pathological condition of the liver tissue from which the cultures were made. Where what grossly appeared as tumors were present in the livers, a comparison was made of the growth and behavior *in vitro* of tissue from the tumors, with liver tissue taken from normal embryonic, normal young, and normal adult rats, respectively. In a few instances tissue from the liver, adjacent to the tumor, was cultured and its growth *in vitro* compared with that of the tumor and with normal control tissue.

MATERIALS AND METHODS

Four series of rats were used in this study. Series 1 consisted only of Wistar strain rats and included 219 animals fed on the dye and 181 fed on dye-free diet as controls; series 2 of 42 Wistar strain rats all of which were fed on the dye diet; series 3 of Wistar strain, Osborne-Mendel strain, and a few Buffalo strain rats, a total of 173 dye-fed animals; and series 4 consisted of 47 Osborne-Mendel strain rats, all of which were dye-fed. All rats used were started on experiment at about 6 to 8 weeks of age, since the death rate for younger rats on the dye was found to be excessive. Approximately 10 to 12 rats were kept to the cage in series 1, 2, and 3, while the number was reduced to about 6 or 8 in series 4. The rats used for tissue cultures were of the Wistar strain.

In series 1 and 2, after an interval of dye feeding, some rats were removed from the dye diet and returned to a dye-free diet. All other dye-fed rats were kept on the dye diet until autopsy. The normal diet consisted of 15.0 percent whole milk powder, 75.47 percent ground wheat, 4.0 percent dried yeast of the Harris and Vitafood brands, 1.4 percent sodium chloride,

0.13 percent ferric citrate, and 4.0 percent cod-liver oil. The 2-amino-5-azotoluene used in the dye diets was originally obtained from Eastman Kodak Co. and was purified by Dr. J. M. Johnson in this laboratory.[1] In the preparation of the dye diet, a 7.5-percent solution of the purified dye was made up in cod-liver oil. A little of this dye crystallized out of the solution on cooling so that the actual stock solution used was slightly less concentrated. Six cubic centimeters of this dye solution was substituted for a like amount of cod-liver oil in each 288 gm. of the stock diet and was thoroughly mixed with it. With rats of series 1, 2, and 3, 288 gm. of this diet was allotted to each cage of 12 rats every 2 days. This allowed each rat 12.0 gm. of food, or approximately 0.019 gm. of dye per day. As some rats died off or were used, the food for each cage was not reduced, with the result that the allotment rose somewhat. The allotment of dye for rats of series 4 was greater than the allotments for series 1, 2, and 3, since rats were kept only 6 to 8 to the cage in series 4, while the amount of dye diet for each cage was held unchanged. Because of wastage, the actual dye consumed in each of these instances was actually somewhat less than that assigned, and in the case of series 4 where the larger amount of food was allotted for each rat a quite substantial amount of food was left uneaten.

All rats for autopsy were decapitated. After gross examination, tissue which showed any abnormality was fixed for study. Some of the liver was always fixed. While at first a number of fixatives were tried, including Orth's, Regaud's, and Zenker-formol, Tellyesniczky's was finally selected and was uniformly used for all of the remaining tissues. Sections were cut 5μ-6μ thick and were stained in Harris' haematoxylin, some with, and some without eosin counterstain. Some sections were also stained in phosphotungstic acid haematoxylin or in Weigert's iron haematoxylin, and a few were treated by the Feulgen technique.

For fixation of cultures for staining, Zenker-formol at 37.5° C. was used uniformly for all except a very few of the early cultures, which were fixed in Orth's fluid, and a few later cultutres which were fixed for the Feulgen reaction. Cultures were stained in Harris' haematoxylin, and a few of the later ones in iron haematoxylin.

In instances where tumor tissue was used for transplantation and for study *in vitro*, the autopsy was done and the desired tissue removed aseptically. The tissue was divided into three parts, one of which was at once cut into suitable pieces and fixed. The part to be used for transplantation was cut up into fragments a little less than 1 mm. in diameter. Twenty to thirty such fragments were drawn up into a 1-cc. tuberculin syringe in approximately one-half cc. of formula No. 11 saline solution and injected, with rigid asepsis, into normal young rats of the same strain.

Tissues excised for culture were generally cut up in the fluid culture medium, although in a few early cultures saline was used. The clumps were at once explanted. Most of the cultures studied were carried in 150 by 16 mm. "roller tubes" (*10*). A few were carried in Carrel D

[1] *In this repurification 500 gm. of the dye was dissolved in hot 95 percent alcohol and decolorized with Carbex decolorizing charcoal. The whole was filtered hot on Büchner funnels; to the filtrate was added an equal volume of water, and the solution was chilled with ice. The crystals that formed were filtered off and dried* in vacuo *over phosphorus pentoxide to constant weight. The 450 gm. of purified dye so obtained had a melting point of 100° C. (uncorrected).*

[2] *This solution consisted of 100 cc. of 70 percent alcohol, 10 cc. of 40 percent formalin, and 5 cc. glacial acetic acid.*

3.5 flasks. Where it was desired to examine the cells at high magnifications or to obtain fixed preparations, tissues were transferred to hollow ground slide cultures.

The tissues were planted in a solid culture medium which was made up of a mixture of equal parts of plasma and a fluid culture medium. In roller tube cultures, 0.6 cc. of plasma was coated over the walls of the tube. An equal volume of fluid medium was added, the whole mixed, and the surplus mixture removed. The tissue fragments were inserted and oriented before addition of the fluid medium to the plasma. In the Carrel flasks, about 1.0 cc. of total solid culture medium was similarly used, and in some instances where a thin clot was desired, after coating the bottom of the flask with this 1 cc. of the medium, some of this clot was removed before gelation. Once the clot had solidified in either roller tube or Carrel flask, 0.7 cc. of fluid culture medium was added and the culture at once stoppered.

About 16 to 20 explants were used in each roller tube; 4 were used for each Carrel flask, while the slides had 1 or 2 each. When liquefaction occurred, a thin layer of fresh clot was added to the culture.

In an attempt to obtain a satisfactory growth of the various types of cells studied, a variety of culture media were used. These are shown in table 1. Either chicken plasma or mixtures of chicken and dog plasmas were used. The dog-plasma addition gave a softer clot which was possibly more favorable to the growth of the epithelial cells. Generally the ratio used was 2 parts of chicken plasma for each part of dog plasma. The chicken plasma contained 0.05 cc. of a $\frac{1}{400}$ solution of heparin for each 20 cc., while the same amount of solution was used for 12.5 cc. of dog plasma. The commercial heparin used was of such purity that 1 mg. was considered sufficient to prevent the clotting of 20 cc. of cat blood for 24 hours.

The fluid culture media generally used contained horse serum, embryo extract, and saline solution (table 1). The horse serum was sterilized by filtration through a Seitz filter under pressure and was stored in rubber-stoppered tubes at 4° C.

Several different saline solutions were tried in an attempt to obtain better growth. Their formulas are given in table 2. Formula No. 11 was most often used, however, and was used for all washing of the cultures. All saline solutions were filtered under pressure and stored in rubber-stoppered tubes. The pH was adjusted with CO_2 to 7.2 before filtration.

The embryo extract used was made from both chicks and rats. The eyes were carefully removed from chicks of 8 days' incubation and the remaining tissue crushed. One volume of chick tissue was used to each volume of saline. After centrifugalization for 30 minutes at about 2,800 r. p. m., and after decantation of the supernatant fluid, a small sterile plug of cotton was inserted into the top of the fluid. The tube was then restoppered and recentrifugalized at 700 r. p. m. The cotton plug was thus slowly driven down through the solution clarifying it of coarse particulate matter and of loose cells which may have remained in the solution. The same procedure was also followed with the rat-embryo extract. After preparation the extract was sealed with cotton and stored in a sealed desiccator, and several breaths of exhaled air were passed into it to keep the pH from becoming too alkaline. The extract was made fresh twice weekly.

Since a constant supply of rat embryos of definite size was not available for the preparation of rat-embryo extract, the embryos used varied somewhat in size. They were used up to full term only when

TABLE 1.—*Medium used in all cultures of rat cells*

Rat No.	Size	Age	Tumor No.	Tube No.	Days used	Culture medium						Total time in cultures
						Parts of saline	For-mula No.	Embryo extract		Horse serum parts	Plasma ratio of chick to dog	
								Total parts	Relative amounts chick and rat			
					EMBRYO RATS							
	Mm.	*Days*										*Days*
1	18			All	To close	8	1	4	3:1	6	4:2	130-37-130.
2	25			All	To close	6	4	3	2:1	4	4:2	88-88.
3	29			All	To close	6	11	3	2:1	4	4:2	73-34-57-73.
4	31			2	8	6	11	3	2:1	4	[1]	
				2	To close	6	11	3	2:0	4	4:2	61.
				3	8	6	11	3	2:0	4	[1]	
				3	To close	6	1	3	2:0	4	4:2	58.
5	31			All	6	6	4	3	2:0	4	4:2	
				All	To close	6	4	3	2:1	4	4:2	54-48-52.
6	37			All	3	6	11	3	0:2	0	4:2	
				All	18	6	11	3	2:0	4	4:2	
				All	To close	6	11	3	2:1	4	4:2	23-23-23.
					YOUNG RATS							
1		35		All	To close	6	11	3	2:1	4	4:2	46-50-36.
2		41		All	6	6	11	3	2:1	4	4:2	
				All	To close	6	11	3	2:0	4	4:2	59-66.
				1	5	7	11	2	2:0	3	[1]	
				1	7	7	11	3	2:0	2	[1]	
				1	To close	6	11	3	2:1	4	4:2	32.
				2	12	7	11	2	0:2	[2] 3	[1]	
3		49		2	To close	6	11	3	2:1	4	4:2	33.
				3	1	7	11	2	0:2	3	[1]	
				3	4	7	11	2	2:0	3	[1]	
				3	7	7	11	3	2:0	2	[1]	
				3	To close	6	11	3	2:1	4	4:2	35.
4		54		All	To close	6	11	3	2:1	4	4:2	15-32.
5		62		All	To close	6	11	3	2:1	4	4:2	14-18-22.
6		69		All	To close	6	11	3	2:1	4	4:2	7-2-7-2-4-2-2-2-2-2-2-7.
					NORMAL ADULT RATS							
1		302		All	To close	6	11	3	2:0	4	4:2	0-0-0.
2		306		All	To close	6	11	3	2:1	4	4:2	8-8-0.
3		311		All	To close	6	11	4	2:0	6	3:1	10-8-8-10.
4		395		All	To close	6	11	3	2:1	4	4:2	0-3.
5		445		All	To close	6	4	3	2:1	4	4:2	3-0-0.
138		706		All	To close	6	4	3	2:1	4	4:2	0-0.
					NONTUMOR AREAS OF DYE-FED RATS							
46		382		1-3	2	5	11	3	2:0	0	[1]	2.
				3	To close	4	11	2	2:0	4	[1]	9.
384		388		All	To close	4	4	2	1:1	4	4:2	0-7.
32		389		All	To close	4	11	2	2:0	4	4:2	20-20.
26		418		All	To close	4	11	2	1:1	4	4:1	9-15-8-14.
33		426		All	To close	4	11	2	2:0	3	4:1	5-5.

[1] Only chick plasma used.

TABLE 1.—*Medium used in all cultures of rat cells*—Continued

TUMOR AREAS OF DYE-FED RATS

Rat No.	Size	Age	Tumor No.	Tube No.	Days used	Culture medium						Total time in cultures
						Parts of saline	Formula No.	Embryo extract		Horse serum parts	Plasma ratio of chick to dog	
								Total parts	Relative amounts chick and rat			
	Mm.	*Days*	1	1	4	5	11	2	2:0	4	(1)	
			1	1	To close	4	11	2	2:0	4	(1)	
46		382	1	2	4	5	11	3	1.5:1.5	4	(1)	22.
			1	2	To close	4	11	2	2:0	4	(1)	
			1	3	To close	5	11	3	2:0	0	(1)	30.
384		388	1–2	All	To close	4	4	2	1:1	4	4:2	4.
32		389	3	All	To close	4	11	2	2:0	4	4:2	9–9.
26		418	7–8–9	All	To close	4	11	2	1:1	4	4:1	21–23.
			1	1	To close	4	11	2	2:0	3	4:1	25–34–17.
			1	3	14	4	11	2	[3] 1:1	4	4:1	47.
			1	3	To close	4	11	2	2:0	3	4:1	
			2	1	To close	4	11	2	2:0	3	4:1	42.
			3	1	5	4	11	2	2:0	3	4:1	92.
			3	1	9	4	11	2	1:1	0	4:1	
			3	1	To close	4	11	2	1:1	3	4:1	
			3	2	5	4	11	2	2:0	3	4:1	94.
			3	2	To close	4	11	2	[4] 1:1	0	4:1	
33		426	3	3	14	4	11	2	1:1	3	4:1	12.
			3	3	To close	4	11	2	2:0	3	4:1	
			3	4	14	4	11	2	1:1	3	4:1	32.
			3	4	To close	4	11	2	[3] 1:1	3	4:1	
			4	1	5	4	11	2	2:0	3	4:1	35.
			4	1	9	4	11	2	1:1	0	4:1	
			4	1	To close	4	11	2	1:1	3	4:1	
			4	3	To close	4	11	2	2:0	3	4:1	
			4	4	14	4	11	2	2:0	3	4:1	94.
			4	4	To close	4	11	2	[3] 1:1	3	4:1	49.
172		428	4	4	To close	4	11	2	2:0	3	4:1	41.
88		444	1–2–3	All	To close	4	11	2	1:1	4	4:1	28–37–28.
			1–2–3–4–7	All	To close	4	11	2	1:1	4	4:1	36–23–22–15–23–36.
22		490	2–3–4	All	To close	6	10	3	2:0	4	4:2	23–23–9–14–26–14–7–7.
95		497	1	All	To close	6	10	3	2:0	4	4:2	7–21–21.
79		524	1	All	To close	4	5	2	1:1	4	4:2	2–2–2–25.
24		531	1–2–3–4	All	To close	6	4	3	2:0	4	4:2	26–37–37–30–2–9–2–37.
			1	1	8	4	11	2		3	4:2	
			1	1	To close	6	11	3	2:1	4	4:2	45.
85		532	1	2	8	4	11	3	2:1	3	4:2	
			1	2	To close	6	11	3	2:1	4	4:2	31.
			1	3	8	4	11	3	2:1	3	4:2	
			1	3	To close	6	11	3	2:1	4	4:2	24.
			1	1–3	To close	4	11	2	2:0	3	4:2	34.
97		532	1	4	34	4	11	2	1:1	3	4:2	
			1	4	To close	6	11	3	2:1	4	4:2	50.
			1	1	To close	4	11	3	0:2	[2] 4	[5] 4:2	18.
93		542	1	2	To close	4	11	3	2:0	4	4:2	26.
			2	1	To close	4	11	3	0:2	[2] 4	[5] 4:2	42.
			2	2	To close	4	11	3	2:0	4	4:2	40.
			1	1	To close	4	11	2	1:1	4	4:2	0.
92		547	2	2	21	4	11	2	1:1	4	4:2	
			2	2	To close	6	11	3	2:1	4	4:2	33.
103		568	1	All	To close	6	1	3	2:1	4	4:2	37–25.
109		568	1–2–3–4.	All	To close	6	1	3	2:1	4	4:2	40–61–14–58–16–16–49.
27		574	1	All	To close	6	4	3	2:1	4	4:2	30–7–23–19–26–23–2–2.
239		588	1–2–4	All	To close	6	1	3	2:1	4	4:2	17–23–29–29–16–38–49.
15		683	1–3–5–6.	All	To close	6	10	3	2:0	4	4.2	58–53–56–53–49–51–56–44.
			1	All	6	6	4	3	2:1	4	4:2	
76		840	1	All	To close	8	6	4	2:0	6	4:1	55–41.
			2	2	6	6	4	3	2:1	4	4:2	
			2	2	To close	8	6	4	2:0	6	3:1	51.

[1] Only chick plasma used.
[2] Rat-embryo extract used with rat serum.
[3] 1 part chick plasma to 1 part rat-liver extract.
[4] 1 part chick plasma to 1 part rat-spleen extract.
[5] 4 parts chick plasma to 1 part rat plasma.

TABLE 2.—*Saline solutions used in cultures of rat tissues*

[Constituents given in grams per liter]

Formula No.	Constituents of saline solution [1]									pH at time of preparation
	NaCl	KCl	$CaCl_2$	$NaHCO_3$	NaH_2PO_4	Dextrose	$MgSO_4$	KH_2PO_4	Peptone (Witte's)	
11	6.8	0.4	0.2	2.2	0.125	1.0	0.10	.....	.....	7.2±
1	6.8	.36	.18	2.0	.14	2.0	.10	.....	.....	7.2±
4	7.5	.36	.18	1.0	.12	1.8	.10	0.03	.....	7.3±
6	7.5	.38	.18	1.0	.12	1.5	.10	.03	5.0	7.4±
10	6.8	.38	.20	2.2	.125	1.0	.10	.03	4.0	7.3±

[1] 1,000 gm. (1 liter) of water used in each solution.

absolutely necessary. Generally they were about 25 to 30 mm. long. For each volume of rat-embryonic tissue 2 volumes of saline were used. The first centrifugalization was carried out at about 700 r. p. m. to prevent haemolysis of the extremely fragile rat erythrocytes. The exact ratios of chick and rat-embryo extracts used for the different cultures are shown in table 1. It will be noted that the ratio generally used was 2 : 1. In a few instances rat-embryo extract was used alone with rat serum. The results obtained were equal to, but not superior to those obtained with horse serum and chick juice. The use of this rat-solution combination was therefore not continued. Extracts of rat liver or spleen were used with a few cultures. These did not seem to give results superior to other combinations and will not be detailed.

All fluid media were changed on the cultures three times a week. Fresh media were added after the culture was rinsed in saline. Whenever the condition of the cultures indicated, they were changed into fresh flasks with fresh culture medium.

OBSERVATIONS AND RESULTS

INFLUENCE OF THE DYE ON THE GROWTH OF THE RAT

Of the rats under experiment, 95 dye-fed rats from series 1 were weighed at monthly intervals in order to ascertain the influence of the dye on the change of weight of the rats. Eighty-four rats from the same series, on the dye-free diet, were used as controls. The dye-fed rats consistently showed less gain in weight than did the controls. This difference continued until, at the end of about 315 days, as an example, 1 group of 23 representative dye-fed male rats showed an average weight of only 247 gm., while a similar group of 36 dye-free controls showed an average weight of 334 gm. The same difference also held for similar groups of female rats.

OCCURRENCE OF PATHOLOGICAL CHANGES IN THE LIVERS OF DYE-FED RATS

Of all rats under experiment in this series of studies, 140 were carried to autopsy. Of these, 105 were of the Wistar strain, 32 were of the Osborne-Mendel strain, and 3 were of the Buffalo strain.

As may be seen from table 3, in so far as could be concluded from gross-autopsy findings, during the first 200 days of dye feeding no tumors were formed in the livers. During the next 100 days, after 201 to 300 days of dye feeding, only 1 gross tumor was found, i. e., less than 8 percent of the rats showed tumors. In the three 50-day intervals from 301 to 450 days of dye feeding, however, the percentages of gross tumors rose sharply to 75, 95, and 79, respectively, while during the last two intervals, from 451 to 614 days of dye feeding, the percentage of tumors reached 100.

TABLE 3.—*Occurrence of tumors of the liver as recognized by gross autopsy findings from 140 rats*

Time interval (in days) after start of dye diet	Total rats (number)	Rats with tumors	
		Number	Percent
0–200	36	0	0.0
201–300	13	1	8.0
301–350	20	15	75.0
351–400	19	18	95.0
401–450	19	15	79.0
451–500	19	19	100.0
501–614	14	14	100.0

These findings are also borne out by the microscopic examinations of 72 livers. The results are shown in table 4. As may be seen from this table, during the first 200 days the sole liver changes seen consisted essentially of injury, regenerative, and early hyperplastic changes. In the interval from 201 to 300 days of dye feeding there occurred only 1 hepatoma and no carcinoma.[3] In the interval from 301 days on, however, there was a sharp rise in the percentage of hepatomata and for the first time, an occurrence of carcinomata. In the interval from 401 to 614 days, of 35 rats examined, only 1 showed changes such as could be considered as merely those of injury, regeneration, or hyperplasia. The remaining 34 rats included 25 which showed hepatomata and 9 which showed carcinomata. Metastases to the lungs were found for 1 carcinoma (rat 40) and 1 hepatoma (rat 92). Typical sections of hepatomata and carcinomata are shown in plates 2, *A* (rat 164) and 3, *A* (rat 26).

Of the various rats studied, No. 33 was of particular interest in view of its later record in tissue cultures. This rat, at 347 dye-days, appeared in a dying condition. Its body was greatly distended with large masses in the liver. All lobes of the liver showed tumor masses, and a number of masses were apparently free in the peritoneal cavity. One mass was attached to the abdominal wall and had invaded the abdominal muscle wall and the diaphragm. The spleen was reduced in size. Four tumorous masses were remove and cultured. Three of these masses were studied microscopically.

Of the four tumors studied *in vitro*, no tissue was fixed from tumor 2. Pathological descriptions of tumors 1, 3, and 4, as detailed by Dr. Hugh G. Grady, are given below:

Rat 33, tumor 1: There is complete disorganization of normal liver architecture and replacement by varying sized nodules composed of enlarged liver cells, sometimes occurring in cords separated by sinusoids but frequently seen in large islands or sheets. Nuclei are of approximately normal size and there are only a few mitotic figures. The cytoplasm, which is slightly acidophilic, varies in consistency from extreme degrees of vacuolation to almost complete homogeneity. There is a marked increase in connective tissue between the nodules and in several areas are seen

[3] *The term "hepatoma" is used in this paper to denote tumors of the parenchyma of the liver, which histologically are not frankly malignant. The criteria for the histological diagnosis of carcinoma consisted of abnormal architecture, of extreme degrees of cellular enlargement, increased range of cell size, great variations in staining reactions particularly for the nuclei, numerous or atypical mitosis, and invasion of the surrounding tissue.*

TABLE 4.—*Character of liver changes as determined from microscopic examination of the livers of 72 rats*

Time interval (in days) after start of dye diet	Total rat livers subjected to microscopic examination	Normal; injury; regeneration; hyperplasia	Hepatoma	Carcinoma	Metastases to lungs
	Number	*Number*	*Number*	*Number*	*Number*
0–200	14	14	0	0	
201–300	3	2	1	0	
301–400	20	7	11	2	
401–500	28	1	22	5	1 (hepatoma).
501–614	7	0	3	4	1 (carcinoma).

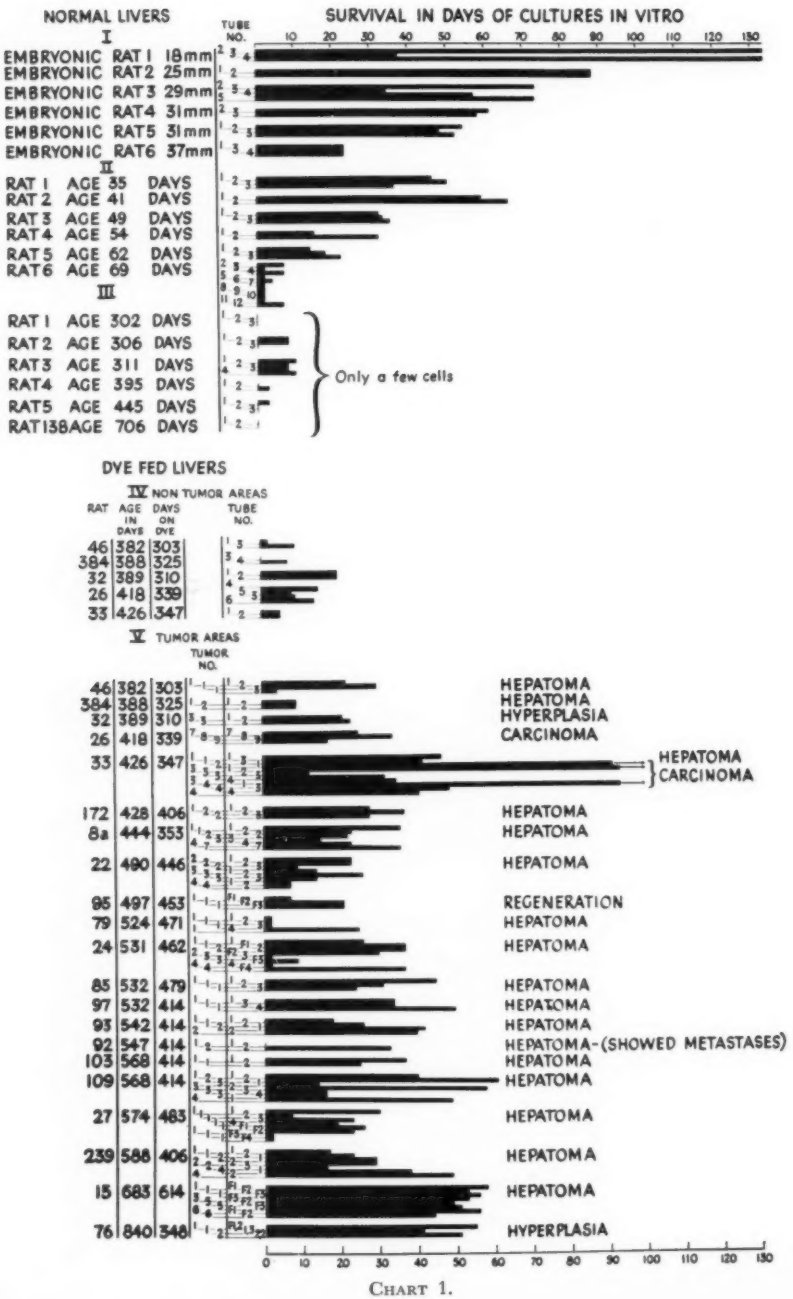
NORMAL LIVERS
SURVIVAL IN DAYS OF CULTURES IN VITRO
TUBE NO.
10 20 30 40 50 60 70 80 90 100 110 120 150
I
EMBRYONIC RAT 1 18mm
EMBRYONIC RAT 2 25mm
EMBRYONIC RAT 3 29mm
EMBRYONIC RAT 4 31mm
EMBRYONIC RAT 5 31mm
EMBRYONIC RAT 6 37mm
II
RAT 1 AGE 35 DAYS
RAT 2 AGE 41 DAYS
RAT 3 AGE 49 DAYS
RAT 4 AGE 54 DAYS
RAT 5 AGE 62 DAYS
RAT 6 AGE 69 DAYS
III
RAT 1 AGE 302 DAYS
RAT 2 AGE 306 DAYS
RAT 3 AGE 311 DAYS
RAT 4 AGE 395 DAYS
RAT 5 AGE 445 DAYS
RAT 138 AGE 706 DAYS
Only a few cells
DYE FED LIVERS
IV NON TUMOR AREAS
RAT
AGE IN DAYS
DAYS ON DYE
TUBE NO.
46 382 303
384 388 325
32 389 310
26 418 339
33 426 347
V TUMOR AREAS
TUMOR NO.
46 382 303 HEPATOMA
384 388 325 HEPATOMA
32 389 310 HYPERPLASIA
26 418 339 CARCINOMA
33 426 347 HEPATOMA CARCINOMA
172 428 406 HEPATOMA
8a 444 353 HEPATOMA
22 490 446 HEPATOMA
95 497 453 REGENERATION
79 524 471 HEPATOMA
24 531 462 HEPATOMA
85 532 479 HEPATOMA
97 532 414 HEPATOMA
93 542 414 HEPATOMA
92 547 414 HEPATOMA-(SHOWED METASTASES)
103 568 414 HEPATOMA
109 568 414 HEPATOMA
27 574 483 HEPATOMA
239 588 406 HEPATOMA
15 683 614 HEPATOMA
76 840 348 HYPERPLASIA
0 10 20 30 40 50 60 70 80 90 100 110 120 150

CHART 1.

small collections of dilated, tortuous bile ducts lined by flattened to low cuboidal epithelium. Diagnosis: hepatoma.

Rat 33, tunor 3: A cellular and vascular tumor mass is seen invading a small fragment of the diaphragm. The tumor is composed of large cells varying in shape, but predominantly polyhedral, and growing as solid sheets or irregular columns separated by sinusoidal vessels. Occasionally tumor cells are seen arranged around a small central space in ductlike fashion. Nuclei are large, hyperchromatic, and mitoses are numerous. Large multinucleated tumor cells are not uncommon and there is a scattering of inflammatory cells in the moderately abundant connective tissue. Diagnosis: carcinoma of the liver.

Rat 33, tumor 4: The section is composed of a vascular tumor composed of large cells resembling liver cells and in general is similar to tumor 3. The tumor cells grow in sheets, irregular columns, and occasionally form ducts or gland-like spaces. In a few areas the cells may even be elongated and spindled. Nuclei are hyperchromatic and vary greatly in size and shape (plate 3, *B*); mitotic figures are numerous. The stroma is abundant and cellular, but there are relatively few inflammatory cells. Diagnosis: carcinoma of the liver

In rat 26 a single tumor was found in one lobe of the liver. This tumor was surrounded by apparently normal liver tissue. This tumor was also diagnosed as a carcinoma and was studied in tissue culture. In section, the cells of this tumor showed many similarities to those of the tumors of rat 33. For instance, the cells were quite irregular in size and shape. The nuclei also were very irregular in size and sometimes showed the extreme distortion and bending seen in the nuclei of the carcinomata of rat 33. What appeared to be budding and amitosis were also observed. In general, although abnormal cell forms occurred, they seemed of less frequent occurrence than in the carcinomata of rat 33.

INJECTION EXPERIMENTS WITH TISSUE FROM DYE-INDUCED LESIONS

An attempt was made to obtain transplantable hepatomata through the injection of tumor tissue from the livers of the various dye-treated rats. From 21 rats with tumors, a total of 150 rats was injected. Of these 21 primary tumors, diagnoses were available on 15. Tumor tissue was injected from these 15 rats into 109 rats.

The results are shown in table 5. As may be seen, tissue from 4 rats in the earlier stages of dye feeding, tissue from 9 rats with hepatomata, and from 2 rats with carcinomata were injected into 15,

TABLE 5.—*Results of in vivo inoculations of liver tumor tissues*

Parent rat Nos.	Diagnoses	Rats injected	Injection site	Results
		Number		
21-32-43-169	Regeneration; hyperplasia.	15	Subcutaneous, intramuscular, intraperitoneal.	Negative.
8A-15-27-35-36-46-57-77-239.	Hepatoma	84	Intramuscular, intraperitoneal, subcutaneous.	Do.
26-33	Carcinoma	10	Intramuscular	Do.

CHART 1.—Survival in days of all rat cultures in vitro.

Section I shows the survival of tissues from livers of normal embryo rats, section II those from normal young rats, and section III those from normal adult rats. Sections IV and V show results from livers of dye-fed rats and include cultures from areas adjacent to gross tumor and from gross tumor areas, respectively.

The survival of the various cultures is shown by the length of the charted lines. Each line represents one tube of cultures. The identification of each individual culture tube is so shown that the medium used in any culture tube may be determined by reference to table 1. In section V the number of the tumorlike mass used from each rat is shown in column 4. Microscopic diagnoses of these tumorlike masses used for culture are shown at the right of the chart.

4, and 10 young rats of the same strains, respectively. Some injections were subcutaneous, some intramuscular, and some intraperitoneal. None of the rats showed tumors at the sites of injections.

CULTURES OF LIVERS, EMBRYONIC, YOUNG NORMAL, AND ADULT NORMAL RATS

The relative longevity *in vitro* of normal liver tissue from rats of different ages is shown in chart 1. Data included are from six series of cultures of embryonic rat livers (sec. I), six series of very young rat livers (sec. II), and six series from the livers of adult normal rats of different ages (sec. III). In all of the instances shown, the cultures were terminated by gradual cessation of growth. With all cultures of embryonic liver there was at first a luxuriant growth. With cultures of the youngest embryo growth continued for about 133 days before loss of the cultures. In the case of the older embryos, growth was of less and less duration as the age of the embryos increased. This lessening of the period of growth continued for the postpartum rats until, with the cultures from livers of all normal rats more than 69 days old, only a few cells wandered out from the explant and the cultures as a whole lived only a few days or else appeared moribund at the end of this time.

In the cultures of the embryonic liver, lobulated sheets of liver epithelium were numerous. These appeared following a preliminary outgrowth of wandering endothelium and fibroblast-like cells. This epithelium was relatively slow in growth. The lobe of a typical living sheet is shown in plate 1, *A*. As may be noted, these cells were more or less polygonal, free from slender processes, and except at the edges of the sheets, adherent on all sides to adjacent cells. The cytoplasm was divided into a granular endoplasm and a clear ectoplasm. The nuclei were ovoid or spherical and contained one, more generally two, rarely three nucleoli. In the replanting of these liver cultures the epithelial-sheet formation was often broken up or obscured by fibroblast-like cells.

Examination of fixed slides available from various cultures of embyronic rat livers showed a few typical epithelial sheets similar in character to those described, for instance, by Doljanski (*11*). In those instances where they appeared, the sheets were very dense and the constituent cells were closely adherent. The cell cytoplasm appeared densely granular throughout the endoplasm. The ectoplasm was practically clear. The nuclei were ovoid or spherical and showed a fairly heavy nuclear membrane; the nuclear substance as a whole showed a diffuse basophilia. There was some granulation in the nucleus. Sometimes one, more generally two, rarely three prominent, rather rounded nucleoli were noted. These stained quite deeply with Harris' haematoxylin and with this stain showed no clear distinction of plasmosome and karyosome. A number of instance of binucleate cells were seen. A typical area of such a fixed and stained sheet is shown in plate 1, *B*.

In some instances, isolated cells or relatively loose sheets of cells very similar to the above were seen, but these could not be identified with certainty as liver epithelium.

While it is obvious that the chief cell present in explants of normal-adult-liver tissue was liver epithelium, no epithelial sheets were ever observed as having migrated out in such cultures. In fact, it was very doubtful if at any time any real migration of epithelial cells occurred. The migration observed seemed to consist solely of wandering cells and fibroblasts. The most common type of cell in all of these cultures of normal rat liver was one which showed the characteristic appearance of the fibroblast. When this

cell showed any sheet formation at all, it was a loose one and was situated at the plasma surfaces. The shapes of these cells varied from very elongate to the very thin sheetlike forms sometimes seen at the plasma surface. The nuclei showed only a very fine granularity which generally did not take the haematoxylin at all except in deeply stained slides. The usual diffuse basophilia of the nuclei above noted was absent. These cells generally showed two rather deeply staining nucleoli, frequently only one, in some instances three or four. In normally stained slides there was no densely stained granulation or reticulum of the nucleus.

GROWTH IN VITRO OF TISSUE FROM AREAS OF LIVER ADJACENT TO TUMORS IN DYE-FED RATS

In five instances of rats which had been fed on the dye, at autopsy cultures were explanted from both the tumor itself and from areas adjacent to the tumor. The survival of these cultures of areas outside the tumor itself is shown in section IV of chart 1 under the heading, Nontumor Areas, while thesurvival of cultures of tumor areas is shown in section V under the heading, Tumor Areas. The general growth of these cultures of nontumor areas was distinctly better than that of the tissue from normal adult rats, but at best the growth was limited and the survival of the cultures short. In every instance the growth of these cultures and their survival were far inferior to those of the cultures from the tumors of the same rat. In the few fixed slides available, cells observed corresponded to the wandering cells or to the cells above described as fibroblasts.

GROWTH IN VITRO FROM TUMOR AREAS OF LIVERS OF DYE-FED RATS

The longevity of cultures from lesions of the livers of 21 dye-fed rats is shown in section V of chart 1. All these lesions on gross examination appeared to be tumors. In these living cultures the outgrowth of cells was irregular. Growth frequently occurred from only a small part of the explant and often not at all. At the first transfer necrotic areas were cut away, and the remaining explant was generally soon surrounded by a vigorous fringe of cells which grew satisfactorily for a time. The longevity of these cultures varied greatly, as may be seen from chart 1. With the sole exception of rat 33, however, within varying intervals up to 62 days from the time of original explantation, all tumor cultures had shown decreasing vitality and had died.

While epithelium was undoubtedly present in the original explants of tumor tissue, characteristic lobulated sheet outgrowths were not always visible. It was observed that many of these sheets were so fragile that they were disrupted when the culture was transferred. The appearance and duration of these sheets varied greatly from culture to culture.

Only a relatively few fixed preparations were made for study of the cells of the hepatomata, since the primary purpose in this work was to obtain growth which would allow the isolation and detailed study of cell strains. Preparations which were fixed and studied showed no clearly defined lobulated epithelial sheets, and no striking changes in cell structure from the cells seen in the normal-liver-culture slides described.

The sole exception to this generalization was 3 strains of cells from tumor No. 33. As may be seen from chart 1 these strains were carried *in vitro* for more than 90 days, and at the end of that time were lost through bacterial infection. The most remarkable feature of these three strains, from the point of view of their growth, was that while all other strains grew well for a time and then died out, these three rat No. 33 strains seemed to become quite

satisfactorily adapted to their culture medium and grew at an excellent rate. At the time these strains were closed out owing to bacterial infection, the original tube 4 strain, for instance, was being carried by luxuriantly growing cultures in 32 tubes. The strain was growing so rapidly that, except for bacterial contamination, it could almost certainly have been run on indefinitely and in a rapidly increasing number of cultures.

In the early stages of these cultures, fibroblasts were present as well as sheets of epithelial cells. The epithelial cells divided rapidly and formed loose sheets of cells in liquefaction areas where they adhered to the glass of the tubes (pl. 4, *A*). On repeated transfer of small clumps from these liquefaction areas, the small colonies of cells, after several weeks' growth produced what appeared to be pure cultures of tumor epithelium which grew to approximately 15 mm. in diameter. Frequently small groups of cells broke away from the parent colonies, floated around in the revolving tubes, and finally reattached themselves and developed into new colonies. The walls of one tube became almost entirely covered with a layer of such tumor cells. The same phenomenon was also seen in slide cultures.

An examination of fixed slides available from cultures of rat 33 tumors showed that in the early stages of their growth these cultures contained many cells of the type described as fibroblast-like. In addition to these, however, there were numerous cells which were manifestly quite different from any so far described. For instance, in an early fixed slide of this series, in addition to what appeared as fibroblasts there was a number of elongated cells with rather deeply staining cytoplasm. The cytoplasm was free from gross granulation. The nuclei were rather elongate and much larger than the epithelial nuclei noted in the cells of normal epithelial sheets. The nuclear membrane was sharply stained. There were two or three large nucleoli in the cells, and they were rather lightly stained and appeared more reddish in color with alum haematoxylin. The nuclei also contained a number of deeply staining chromatin granules.

In the later stages of these strains there were fewer fibroblast-like cells. This may in part have been due to an attempt to select out and carry on the cultures which seemed to be largely cells of the type described and to close out those cultures in which fibroblast-like cells were more numerous. Still later, that is, at about 80 days after explantation the cultures seemed entirely free from the fibroblast-like cells. To all appearances the cultures were a pure strain of tumor epithelium.

The variety of forms assumed by these cells was very great. The cells were sometimes in quite typical epithelial sheets (pl. 4, *B*). At other times the cells were widely separated from one another. As a rule they were rather rounded, angular, or lobular in outline, although a few with very slender processes were seen. Frequently the growth was that of a very loose sheet of cells. With the passage of time the irregular shape of the cells and the nuclei became increasingly prominent in the cultures. Many cells showed quite elongate nuclei. Other nuclei were twisted, hook-shaped, or lobulate (pls. 4, *B*, and 5, *A*); still others showed what appeared to be buds. Nuclei which looked as though they were undergoing cleavage by amitosis were common (pl. 5). The variation in internal nuclear structure was equally great. In some instances the nuclei showed only a clear diffuse basophilia with the aluminum haematoxylin. As a rule the nuclei showed a number of relatively deeply stained chromatin granules or a partial coarse chromatin net.

The nucleoli were generally lightly stained and often appeared as practically achromatic plasmosomes. Sometimes, however, they stained quite deeply and uniformly (pl. 5, *A*).

The sizes of these cells and their nuclei also showed a very great increase in range. Some cells were the size of the normal cells described as liver epithelium. Others were much larger. For instance 1 cell was found in which the width of the nucleus was 8 times normal while the length was 10 times normal length. In this cell there were several hundred chromatin granules in the nucleus and probably about 100 nucleoli. These nucleoli showed typical plasmosomal staining reactions. Cells which contained 2 or more nuclei were frequent. Cells which showed mitosis were so frequent as to indicate a rapid rate of growth of the culture.

No striking differences were noticed between the epithelial cell strains from tumors 2, 3, and 4 from rat No. 33. Too few slides from tumor 1 were available to allow a detailed comparison. The living cultures of this strain were recorded as containing a great number of fibroblasts and for this reason the strain was shortly closed out, with the exception of one tube which died out at 47 days.

The similarity of these tumors, from rat No. 33, in their cellular morphology *in vivo* and *in vitro* is obvious from a comparison of plates 3, *B* and 5, *A*.

DISCUSSION

In this series of studies, the aim of the dye-feeding experiment was to produce liver tumors as rapidly as possible, and at the same time to maintain the rats on a balanced diet in order to rule out any complications from dietary imbalance. Since seemingly satisfactory concentrations of dye in the diet had already been worked out by Sasaki and Yoshida (*3*), these were accepted; but instead of a diet of polished rice, a balanced mixed diet was used.

While the approximate amount of dye assigned to each group of rats is known, the actual amount of food eaten by each rat and consequently the exact amount of dye each rat received, are not known. For this reason the retarded gain in weight of dye-fed rats while on the dye diet cannot be assigned entirely to the systemic action of the dye. Some of this lack of gain of weight may, and probably did, arise from the fact that the rats on the dye diet did not like the diet and had the tendency to scratch it out of the feed cups and waste it.

The action of 2-amino-5-azotoluene in the production of liver tumors was extremely slow. The first tumor, a hepatoma, made its appearance in a rat autopsied 211 days after the commencement of the dye diet. The first tumors which developed were isolated single masses surrounded by what appeared grossly as normal tissue. Autopsied animals fed for long periods on the dye diet showed multiple tumors which frequently involved the entire lobe or all lobes of the liver.

In comparison with the results obtained in this study, Sasaki and Yoshida (*3*) obtained tumors in a minimum of 96 dye days, while the maximum time was 255 to 300 dye days. Kinosita (*9*) also used the polished-rice diet and found that in many of his rats a period exceeding 400 days was required for the production of tumors. As may be seen, the results reported in this paper fall between those of Yoshida and Kinosita in point of time required. Some differences could probably have risen from differences of rat strain or of diet.

The individual variation in the time required for production of hepatoma or carcinoma through the action of the dye is very clearly shown, for instance, by com-

parison of the records of rats 33 and 15. In the instance of rat 33, the liver had undergone carcinomatous changes in 347 days, while the liver of rat 15 had only undergone hepatomatous changes in 614 days. The mechanism of this difference is not indicated by the data.

Of the various tumors which were examined microscopically, 11 were histologically classified as carcinoma. In the instance of rat 33, definite invasion of the abdominal wall was observed. In the instances of rats 40 (carcinoma), and 92 (hepatoma) metastases to the lungs were found (pl. 2, *B*). With these findings there seems no question but that actual carcinomas were produced. Zylberszac (*4*) also found pulmonary metastases.

At the start of the work with tissue cultures, the first medium tried was one already available in this laboratory. It consisted of a mixture of horse serum, chick-embryo extract, and saline. This medium was tried with rat embryonic liver. Because of the toughness of the avian-plasma clot some trouble was experienced with this medium in the handling of the delicate epithelial sheets. In order to eliminate this difficulty a small percentage of dog plasma was added to the avian plasma. The result was a softer clot which seemed easier to handle.

With the hope of further stimulating growth, homologous (rat) embryo extract was next tried in the culture media. With the mixtures used difficulty was experienced in obtaining a rapidly clotting mixture, as the combination of rat-embryo extract and horse serum seemed to reduce the clotting rate. Rat serum was therefore substituted for the horse serum. With this combination a satisfactory clotting time was obtained, but since growth was no better with rat-embryo extract and rat serum than it was with chick-embryo extract and horse serum, the rat solutions were abandoned. The mixture finally adopted as one easily handled and quite as satisfactory consisted of a mixture of chicken and dog plasmas, chick-embryo extract, and horse serum, diluted with saline.

It may be noted that a number of changes were made in the saline solutions used. As may be seen from table 1, the one originally used was the formula designated in this study as No. 11. This saline was already in use in this laboratory and was originally described by Earle (*12*). This solution was used continuously for washing all cultures and for preparation of all extracts. After several changes this formula was finally adopted with the addition of 4 gm. of Witte's peptone in each liter.

Several attempts were made to grow tissue without serum, but no success was experienced. One or two attempts were made to use extracts from spleen and liver but these did not prove superior to chick-embryo extracts.

A comparison might be made of these results with those of other workers. Morigami (*13*), using rat-embryo extract and rat serum, succeeded in growing the cells of induced rat hepatomata for about 22 days. Doljanski (*11*), working with the livers of 8- to 10-day-old chick embryos and using mixtures of plasma and heated chick-embryo extract, obtained pure cultures of liver epithelium which he carried for more than 120 days. Nordmann (*14*), using plasma and a Locke-Lewis solution, sometimes with embryo extract, grew normal embryonic rat liver and young rat liver for intervals up to about 15 days.

In examining the behavior of the cultures in the different types of culture media used, within the range of conditions, except in those instances where serum was omitted, the selection of the culture media seemed to have little actual influence on the growth

of the cultures. The major influence controlling the growth of the various cultures studied seemed to be the intrinsic differences in the nutrition of the various types of cells. For this reason the results obtained from the use of this variety of culture media have been grouped in chart 1 for approximate comparison.

From this chart it is obvious that the normal embryonic liver of the youngest rat used could grow for quite an extended time (133 days). With increasing age of the rat this growth decreased sharply, until with the adult rats, no epithelial growth was noted. That this decreased potentiality of growth of the liver cell under the conditions used was owing to progressive differentiation of the cells in the developing normal liver seems quite probable. The mechanism of this decreased potentiality was not indicated by data obtained.

With the production of tumors in the liver, areas of tissue taken from close to, but outside the active-tumor areas, showed a slight increase of growth over the normal adult-liver controls. Even this growth was of relatively loose architecture and was rather sparse. It could not be identified as epithelial in nature, and may have been solely, or in large part, fibroblastic or endothelial. It is suggested that this growth may have resulted from slight injury, or from the action of the dye with consequent regenerative stimulation to the nontumorous areas of the liver.

There seems no question, however, that in the dye-fed rats shown in chart 1, there was a great increase in the growth and longevity of many of the cultures from the various tumors. In instances the cells appeared as recognizable sheets of liver epithelium. In all but the tumors of rat 33, however, it appeared that these hyperplastic, hepatomatous, and even carcinomatous cells had not been sufficiently altered to allow their continued growth under the conditions of culture used. The nutritional requirements of these various neoplastic cells is in strong contrast with those of cells of the carcinomata of rat 33. These carcinomatous epithelial cells from rat 33 were obviously so radically altered in their nutritional requirements that they lived and grew luxuriantly in excess of 90 days. At that time, if infection had not supervened, it is practically certain that they could have continued to grow luxuriantly, under the conditions used, for an unlimited time.

These data represent an extension and confirmation of data previously presented by Earle (*15*) for the mammary gland. Earle showed that while the normal mammary-gland cell could not live and grow satisfactorily under the conditions imposed, the malignant cells of the Walker 256, which arose from the mammary-gland epithelium, were so radically altered in their nutritional requirements that they could live and grow indefinitely in the same culture medium.

Chart 1 shows that of the 10 tubes of cultures started from 3 different tumors of rat 33, some grew for extended periods while others did not. Similar differences in growth were also frequently seen for cultures from other tumors, as, for example, for the cultures from the tumors of rat 24. Some of this variation in longevity of the cultures almost certainly arose from minor differences in the handling. For instance, with cultures which are growing relatively slowly, a very careful trimming is necessary in order to get a satisfactory growth on transfer to a fresh tube; while with rapidly growing cultures, slight errors in trimming are of little import. With the majority of those cultures studied in this work, a slight error could easily have resulted in the complete loss of the culture. Some losses of cultures from this and similar causes almost certainly did occur.

Another source of variation in culture longevity was apparently local variations within the tissue itself. For instance, in sections of primary tumors examined, one area sometimes showed normal liver tissues; another region very close showed a hepatomatous transformation and in a very limited area showed what was microscopically carcinoma. Since many of these transitions were not discernible on gross examination, and since tissue for culture was chosen from gross examination, there was much variation in tissue used for the different explants. Where there was great variation in the cultures from any one tissue, it is probably more accurate to leave out of consideration the cultures which lived a relatively short time and to consider those which lived for longer intervals as representative of the more anaplastic areas of the tissue grown.

This local variation in the tissue selected probably accounts for the fact that the tumor from rat 26, although in some areas diagnosed carcinoma, grew for only a limited interval of time. Very probably the actual tissue area used for culture was not from an area of such carcinomatous change. The possibility cannot be excluded, however, that while the tissue was morphologically carcinoma, it had not yet reached a stage of nutritional change which would allow its cells to live indefinitely under the conditions used.

Not only were the nutritional characteristics of the cells from the tumors of rat 33 radically different from the normal epithelium and from the epithelia of the dye-induced hepatomata studied *in vitro*, but the cells themselves were radically altered morphologically. This may be, clearly seen by comparing plate 1, *B*, with plates 4, *B*, and 5, *A*, *B*, and *C*. The nuclear changes seem particularly remarkable. Instead of the ovoid or spherical nuclei of the normal cells, the nuclei were quite elongate, varied greatly in shape and in size, and showed a distinct tendency to elongate curve shapes. Numerous distortions of shape and size strongly suggested amitotic nuclear division and nuclear budding. The increased variability of nuclear size was also prominent in the cells of tumors of rat 33. The strong similarity of some of the distorted, lobulated, irregular nuclei of the cultures to those of some of the sections of the tumor *in vivo* may be easily seen by a comparison of plates 3, *B*, and 5, *A*.

No satisfactory explanation can be offered for the negative results from the attempts to induce transplantable hepatoma by injection of tissue from dye-induced tumors, except that, as is well known, first generation implants of tumor tissue are often very difficult to start growing.

SUMMARY

The effect of the oral administration of the dye 2-amino-5-azotoluene on young rats of the Wistar, the Osborne-Mendel, and the Buffalo strains was studied over periods of dye feeding up to 614 days.

The action of the dye on the increase of weight of the young rats to which it was administered was such that dye-fed rats weighed over a period of 10 months showed much less increase than did normally fed controls.

One hundred and forty dye-fed rats were autopsied after various intervals of dye feeding. In these rats the first gross tumor was observed at 211 days of dye feeding. The percentage of gross tumors increased sharply from 300 days on; in the interval from 301 to 614 days of feeding, 81 of 91 rats showed gross tumors, while of 33 rats examined from 451 to 614 days of dye feeding, all showed gross tumors.

Microscopic examination of 72 livers

showed that for the first 210 days of dye feeding, changes in the liver were those of injury, regeneration, and hyperplasia. At 211 days hepatomata first appeared, and these became increasingly numerous in later periods of dye feeding. From 401 to 500 days of feeding, 22 of 28 rats examined showed hepatomata. Carcinomata first appeared in the interval from 301 to 400 days, and increased in number until, in the interval from 501 to 614 days, of 7 rats examined 4 showed carcinomata. Eleven carcinomata were observed in all. Two instances of metastases to the lungs were found.

The results from the injection of tissue from 9 of these dye-induced hepatomata into 84 rats, and the injection of tissue from 2 carcinomata into 10 rats were all negative.

The growth *in vitro* of liver tissue from normal embryonic, normal young, adult rats, and also tumor tissue and tissue from adjacent liver areas of dye-fed rats of various ages were all studied in a variety of combinations of culture media. As long as serum was included, little difference was noted in the growth-promoting actions of the various culture media tried. There was, however, a marked difference in the growth potentialities of the different types of tissue used.

While the early embryonic tissue showed an excellent growth of epithelium, the cultures from even the youngest rat embryo used lived only 133 days. As the age of the rat increased, the survival time of the cultures lessened until, with the normal adult rat, there was no real epithelial growth at all and usually little or no fibroblastic growth.

Areas of tissue adjacent to tumors taken from livers of dye-fed rats showed a slight increase in growth over tissue of normal controls, although this growth seemed limited to fibroblast- and endothelial-like cells.

Cultures from dye induced hepatomata, and from one carcinoma (rat 26) showed a great increase of growth over the normal-liver controls. The increase of growth involved the epithelial cells, but continued for a limited time only, and at the end of 61 days after explantation all cultures had died. It is pointed out that as a result of local variation within the tumor the cultures from the carcinoma may not have included any actual areas of carcinoma but only less radically changed cells.

One carcinoma (No. 33) showed very excellent growth *in vitro*. This continued for 94 days at which time the cultures were lost because of an infection. The cells of this carcinoma grew luxuriantly and showed every evidence of satisfactory adaptation to their *in vitro* medium. There can be little doubt that this strain could have lived on indefinitely if infection had not supervened. Fibroblasts and other cells gradually disappeared from these cultures until there was finally left what appeared as a pure strain of epithelial cells.

The cells from rat No. 33 were obviously greatly altered nutritionally and morphologically from the hepatic epithelium from which they arose. The cultures of these cells are described *in vitro*, and their morphology *in vitro* is compared with that *in vivo*.

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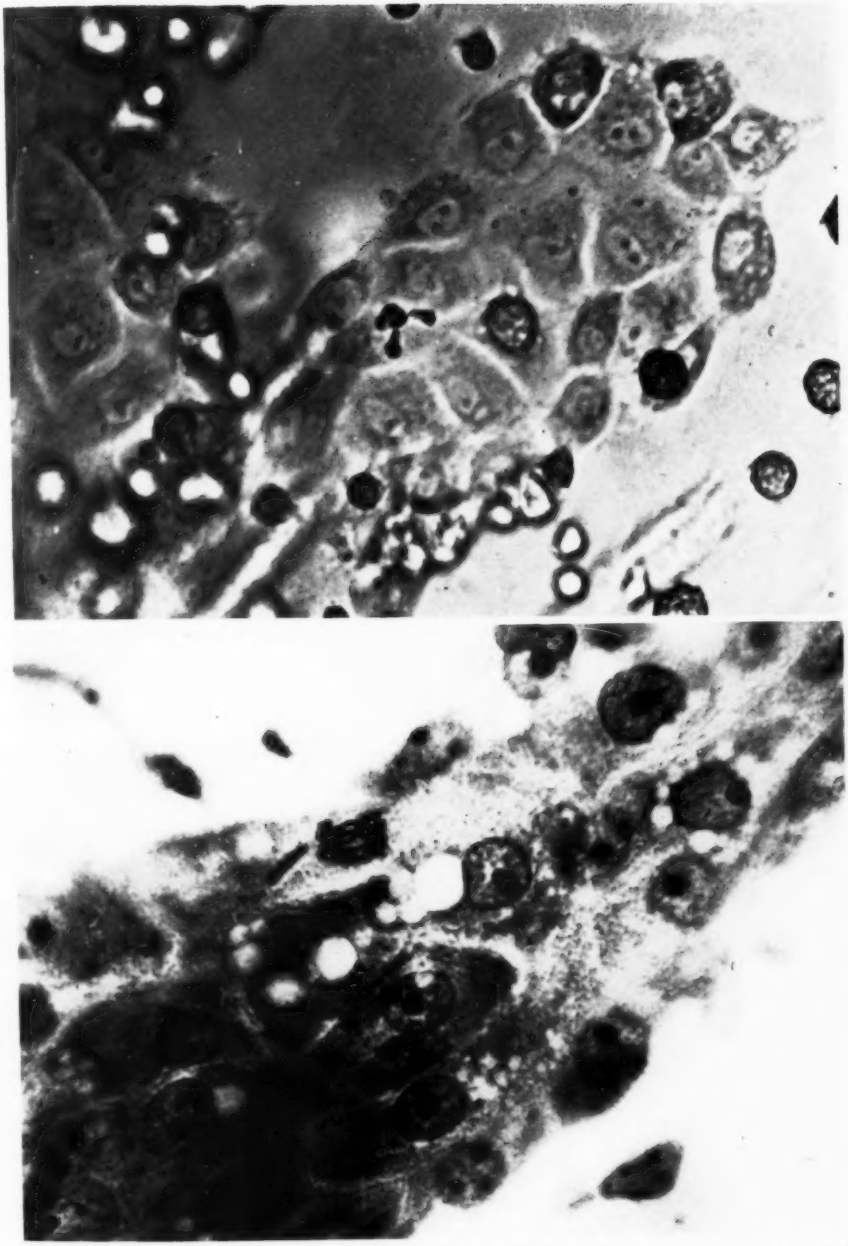
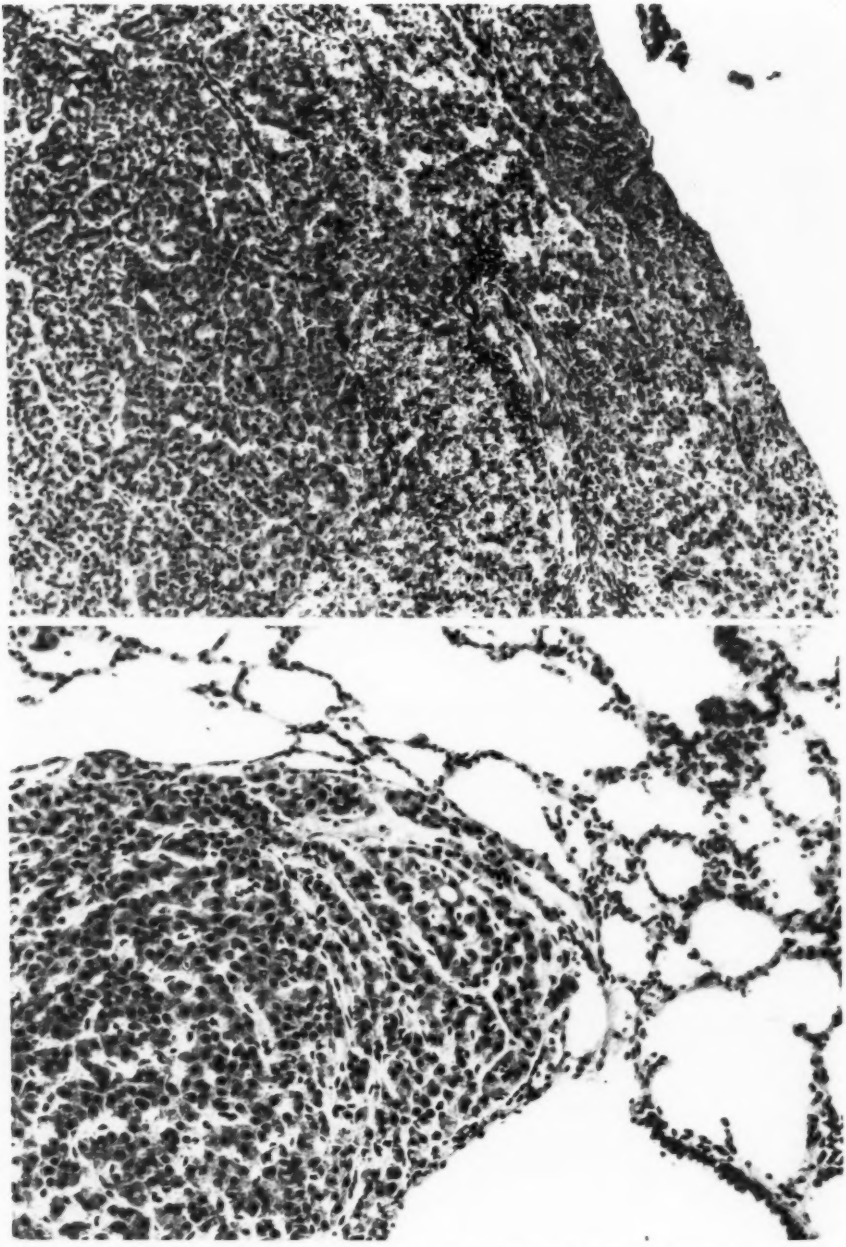
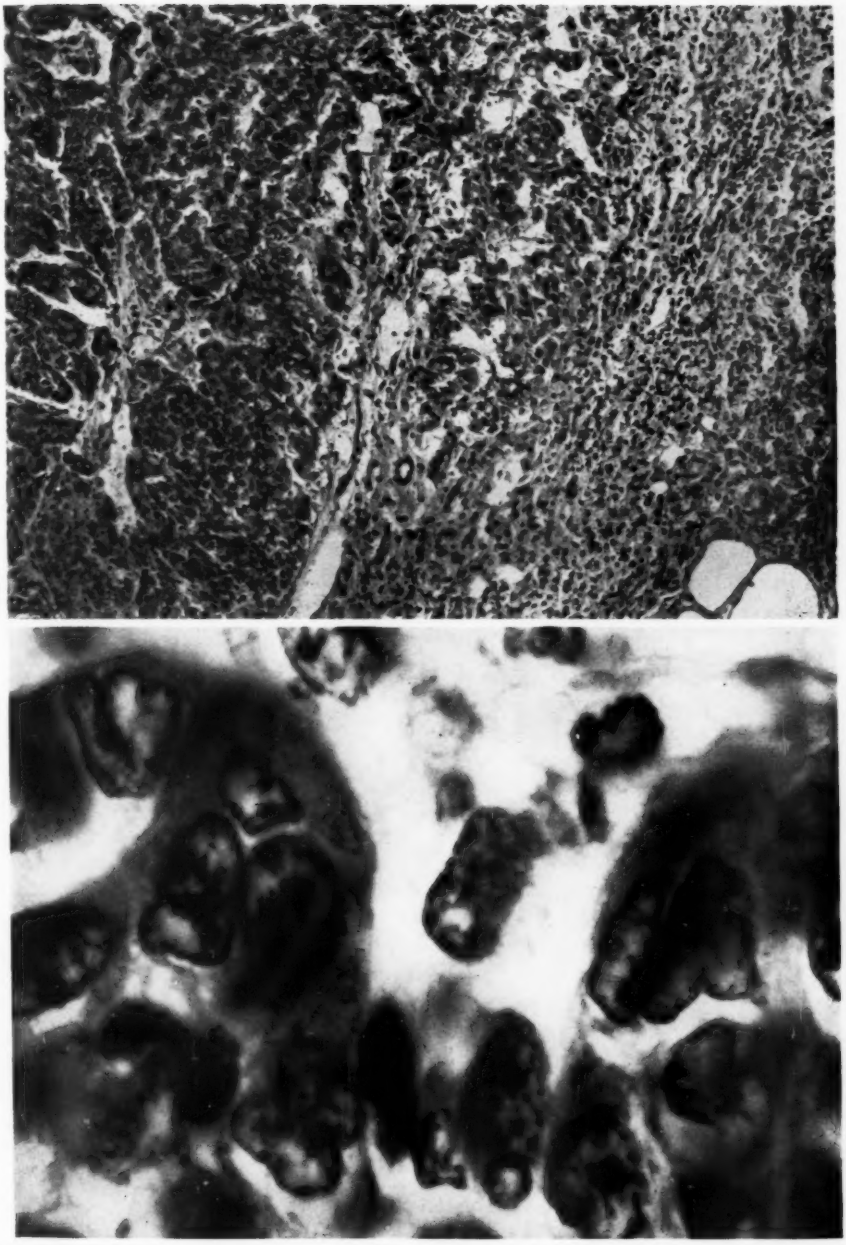


A, Typical normal living lobulated epithelial sheet from the liver of embryonic rat No. 3, 20 days after explantation. Note characteristic epithelial cells. *B*, Typical epithelial sheet from the liver of embryonic rat No. 5. Photographed from fixed and stained preparation. Note granular cytoplasm and characteristic nucleus. Heidenhain's haematoxylin.

PLATE 2

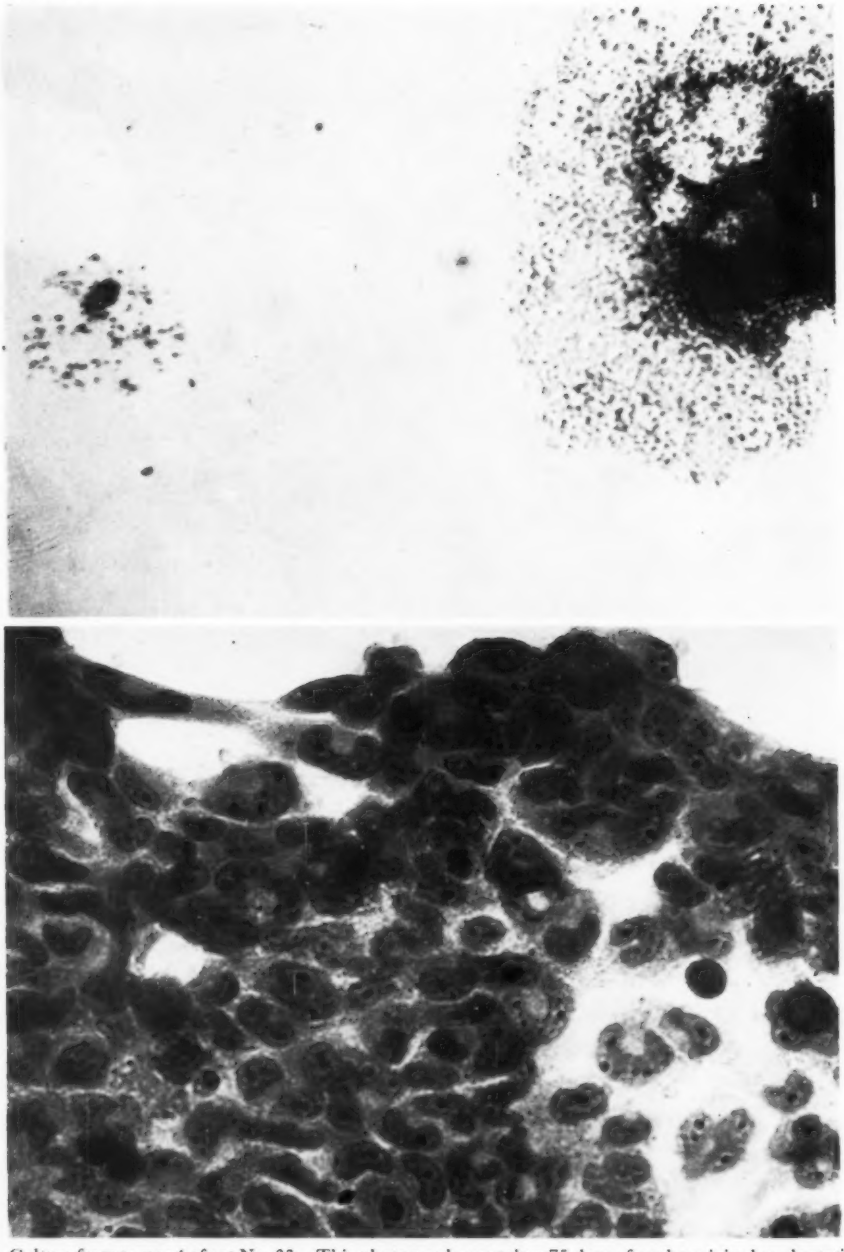


A, Typical section from a hepatoma taken from rat No. 164. Note the relative similarity of the hepatoma tissue and the adjacent area. Compare with the invasive growth of the carcinoma of rat No. 26, plate 3, *A*. Stained with haematoxylin and eosin. × 124. *B*, Metastases in lung from carcinoma of rat No. 40. × 177.



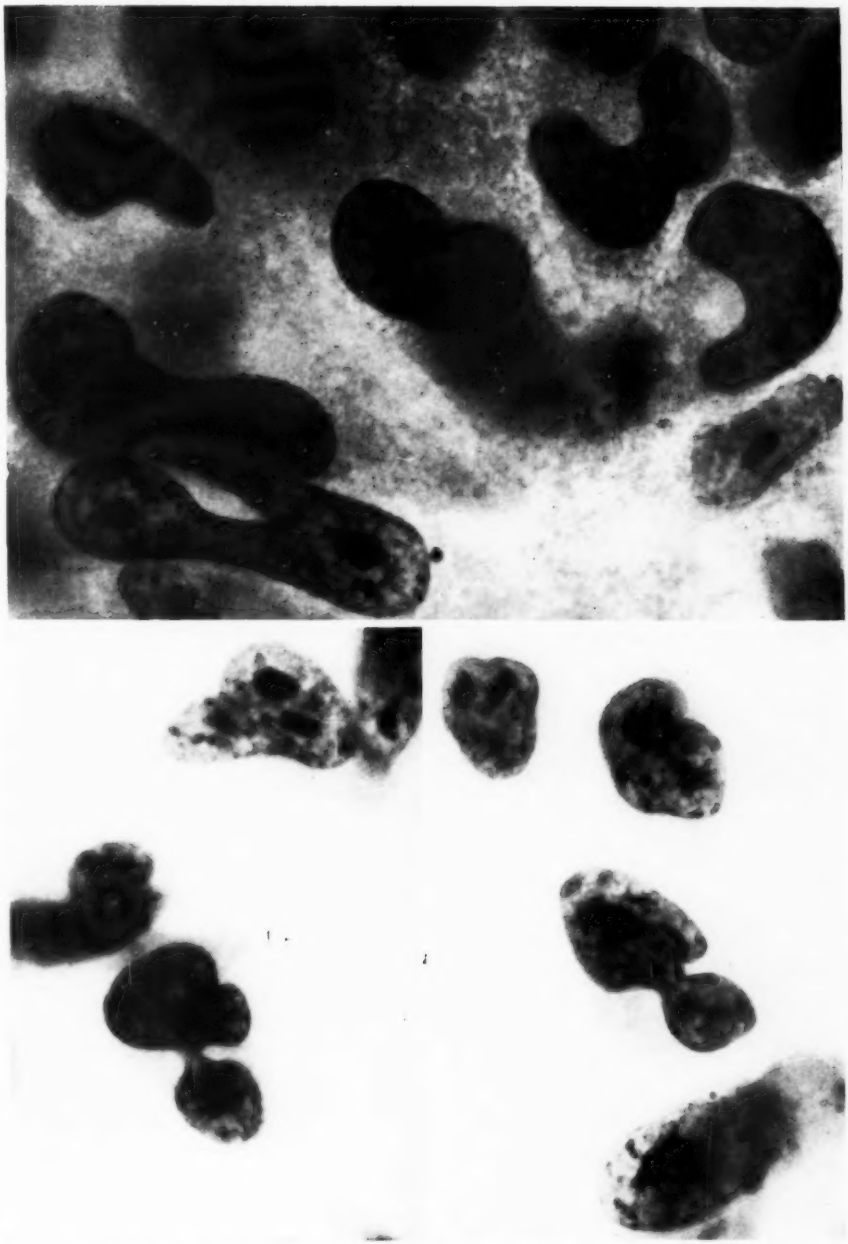
A, Section of carcinoma of rat No. 26. Note the invasion of the adjacent area by the carcinomatous tissue. Stained with haematoxylin and eosin. × 122. *B*, Portion of tumor 4 of rat No. 33. Note the very distorted, convoluted forms of nuclei seen. (Compare with plates 4, *B*, and 5, *A*.) × 1772.

PLATE 4



A, Culture from tumor 4 of rat No. 33. This photograph was taken 75 days after the original explantation of the tumor and 3 days after transfer of the culture. Note the characteristic loose epithelial sheet of cells, also the start of a new culture from cells which have come loose from the parent colonies. × 44.

B, General view of a fixed and stained epithelial sheet from a culture of tumor 4 of rat No. 33 Compare with the normal epithelial cells shown in plate 1, *A* and *B*, and with the cells of tumor 4 of rat No. 33 *in vivo*. Note the general curved shape of the nuclei. Fixation, Zenker-formol. Stained with Harris' haematoxylin. × 443.



A, Higher power view of the same sheet from tumor 4 of rat No. 33. × 1772. Note the general character of the nuclei and compare with plate 3, *B*. *B*, and *C*, Fixed and stained preparations from culture of tumor 4 of rat No. 33. Two areas selected to show extreme distortion of cell nuclei, with suggestion of amitosis. × 1545.

SQUAMOUS CELL CARCINOMA AND OTHER LESIONS OF THE FORESTOMACH IN MICE, FOLLOWING ORAL ADMINISTRATION OF 20-METHYLCHOLANTHRENE AND 1,2,5,6-DIBENZANTHRACENE

(Preliminary Report)

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In a previous communication, experiments were described in which adenocarcinoma of the small intestine and other lesions were obtained by the oral administration of aqueous olive oil emulsions containing either methylcholanthrene or dibenzanthracene (Lorenz and Stewart (*1*)). Strain A and strain A backcross mice were used. In the experimental animals employed, no lesions of the stomach were observed. The duration of these experiments was between 7 and 13 months.

New experiments along these lines were carried out. Mice of strains A, C57 brown, C57 black and C_3H were given orally an aqueous olive oil emulsion of either methylcholanthrene or dibenzanthracene. Mice of strain A backcross were given the same olive oil emulsion to which 0.5 percent of the dioctyl ester of sulfosuccinic acid (trade name: Aerosol OT) was added for stabilizing purposes. A third experiment consisted in administering orally to strain A backcross mice an aqueous mineral oil emulsion containing either methylcholanthrene or dibenzanthracene. These experiments have been in progress now for 7 months, and the daily intake of emulsion has been approximately the same as in the previous experiments (Lorenz and Stewart (*1*)).

In the animals which have come to autopsy up to the present time, there have been observed the following lesions of the forestomach in 40 animals: hyperkeratosis and hyperplasia of the mucosa (6 mice), single or multiple papillomas (24 mice), and squamous cell carcinoma (10 mice). In several animals all combinations of these lesions were present. In addition 13 of the animals with changes in the stomach had adenocarcinoma of the small intestine. The gastric lesions occurred earliest, in greatest proportion, and were largest in the mice ingesting the methylcholanthrene emulsions, and especially the one prepared with mineral oil. All the gastric carcinomas to date have occurred in the strain A mice and strain A backcross mice ingesting methylcholanthrene emulsions.

The mice with hyperplasia and hyperkeratosis of the mucosa of the forestomach exhibited the lesion either in localized areas or diffusely. Microscopically, the change consisted of thickening of the squamous epithelium with a large amount of keratin on the surface and sometimes fibrosis and hyalinization of the submucosa.

The single or multiple papillomas observed in the forestomach were filamentous, sessile, or cauliflower in type. All these were confined to the cardiac chamber and were so numerous in some instances that practically the entire mucous membrane was involved. Microscopically, they consisted of a central core of connective tissue, containing blood vessels and covered with hyperplastic and hyperkeratotic squamous epithelium. In some papillomas there were changes suggestive of early malignancy.

The majority of the stomachs with squa-

FIGURE 1.—Sections of a stomach, showing marked narrowing of the lumen of the cardiac chamber and infiltration of the wall by squamous cell carcinoma. Photograph of gross specimen, circa × 1.5.

mous cell carcinoma were greatly enlarged, and a palpable mass could be outlined during life in the region of the stomach. The forestomach was enlarged several times, and the pyloric chamber was small. On the serosal surface, there were usually one to several nodules of varying size. In some cases, these nodules extended directly into the spleen, diaphragm, body wall, and pancreas. In several cases there were grayish masses in the mesenteric lymph nodes, and in one case each a small gray nodule in the genital omentum and in the lung. Upon opening the stomach after fixation, the wall of the forestomach was found to be markedly thickened, either diffusely or in localized areas, by grayish-white tissue which in many cases almost completely obliterated the lumen (fig. 1).

The mucosa of the pyloric chamber was not involved except in certain cases in which the cardiac mass overhung somewhat the glandular mucosa. Microscopically, all the carcinomas were of the squamous cell type and showed extensive keratinization (fig. 2). The tumor tissue extended through all coats of the gastric wall, formed nodular masses on the peritoneal surface, and in three cases showed infiltration of, or metastases to the mesentery, pancreas, mesenteric lymph nodes, genital omentum, spleen, diaphragm, lung (fig. 3), chest wall, and posterior abdominal wall.

No case of spontaneous squamous cell carcinoma of the forestomach in mice of the strains used in these experiments has occurred in this laboratory.

Conclusions: These experiments illus-

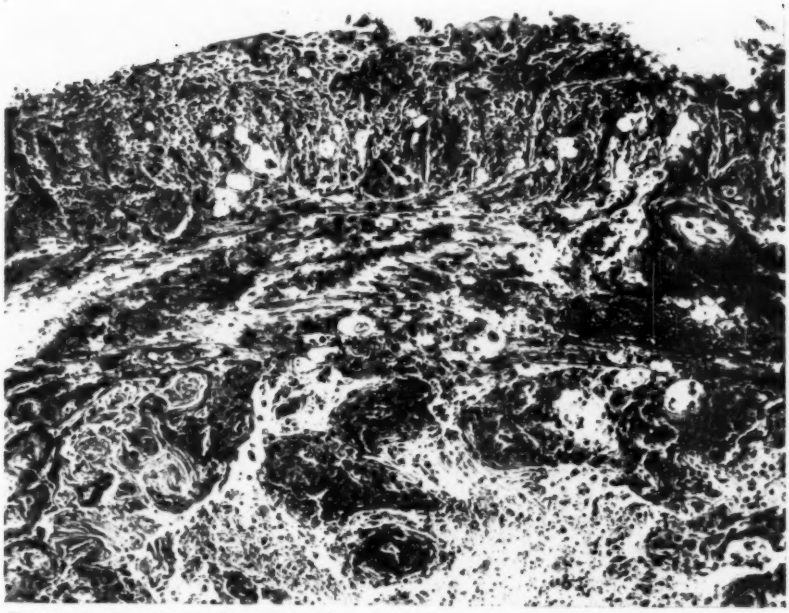

FIGURE 2.—Stomach wall above and lumen below. All coats of wall are infiltrated by squamous cell carcinoma. Photomicro × 88.

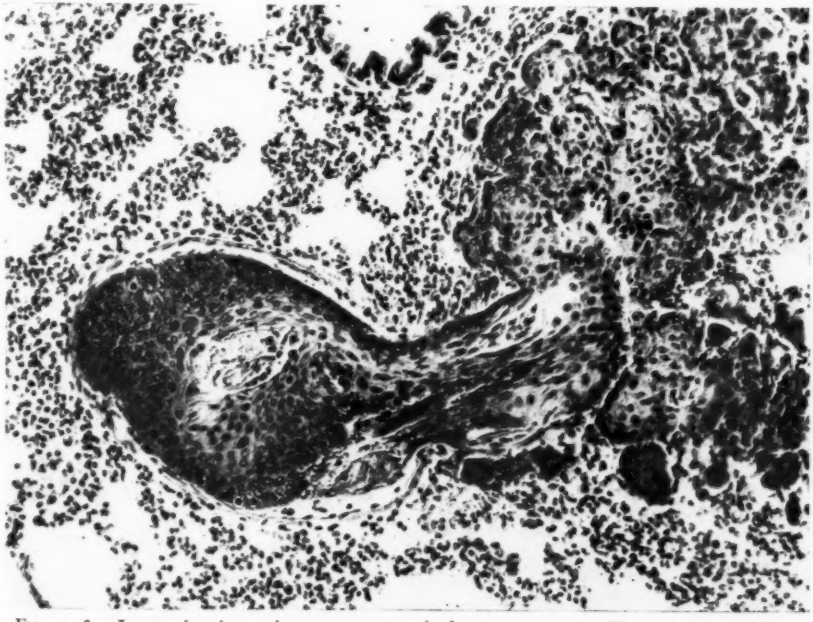

FIGURE 3.—Lung, showing pulmonary metastasis from squamous cell carcinoma of forestomach. Photomicro × 183.

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trate that the induction of squamous cell carcinoma of the forestomach of mice depends in part upon the selection of the medium in which the carcinogenic hydrocarbon is administered and in part on the strain of mouse used.

REFERENCES

(*1*) LORENZ, E., and STEWART, H. L.: Intestinal carcinoma and other lesions in mice following oral administration of 1, 2, 5, 6-dibenzanthracene and 20-methylcholanthrene. J. Nat. Cancer Inst., **1:** 17–40 (1940).

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